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Award Number: DAMD17-01-1-0045

TITLE: Overcoming Resistance of Prostate Cancer to TRAIL-Mediated Apoptosis

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REPORT DATE: June 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2003	Annual (1 Jun 2002 - 31 May 2003)

4. TITLE AND SUBTITLE
Overcoming Resistance of Prostate Cancer to TRAIL-Mediated Apoptosis

5. FUNDING NUMBERS
DAMD17-01-1-0045

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Purpose – TRAIL (TNF-related apoptosis inducing ligand) is a protein that induces apoptotic cell death in cancerous but not normal tissues. TRAIL has the potential to be a novel prostate cancer treatment. However, we have found that the majority of prostate cancer cell lines are insensitive to this agent. The goal of this proposal is decipher the nature of this resistance and develop biochemical tools to enhance the sensitivity of prostate cancer to TRAIL killing.

Scope- This proposal examines normal prostate epithelia, prostate cancer cell lines, and animal tumors for sensitivity to TRAIL.

Major Findings- We find that prostate cancer cells can be induced to undergo apoptosis when the proteasome inhibitor, PS-341 is added to TRAIL. PS-341 overcomes TRAIL resistance in prostate cancer cells that are bcl-xL overexpressors, as well as other cells that are Bax or caspase 9 negative. TRAIL/PS-341 killing require the presence of Bak in the tumor cells to induce cell death.
Results and Significance- These findings suggest that this combination may be viable as a treatment regimen. Our results demonstrating that cytotoxic TRAIL monoclonal antibodies also synergize with PS-341 suggests that two agents under clinical investigation can be combined to kill prostate cancer.

14. SUBJECT TERMS
TRAIL, Apoptosis, AKT, Protein Kinase, Vitamin D

15. NUMBER OF PAGES
63

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Subject- TRAIL (TNF-related apoptosis inducing ligand) is a protein that trimerizes its receptor and activates caspases to induce apoptotic cell death. We have shown that although some prostate cancer cell liens are extremely sensitive to TRAIL, most are either

Partially or completely resistant. Because TRAIL does not kill normal cells but does kill tumor cells, if the resistance could be overcome, this would be a potentially important anticancer agent. However, TRAIL protein has been shown to have liver toxicity when incubated with human liver cells. This result suggests that the clinical use of this agent could be complex. The purpose of this grant is to (1) identify the mechanism of resistance of human prostate cancers to TRAIL-induced apoptosis; (2) Determine whether the sensitivity of normal tissues to TRAIL depend on the levels of AKT activity; and (3) Determine how calcitriol functions to modulate TRAIL-induced apoptotic killing. The scope of the research involves studying normal prostate epithelia in tissue culture, malignant human prostate cancer cell lines, and nude mice with subcutaneous tumors to validate these hypotheses.

BODY

Task 2- The goal of this Task was to quantitate the binding of specific proteins to TRAIL receptor under varied conditions. The data collected in this task are in publication form under review at the Journ. Biol. Chem (publication 2). TRAIL does not kill normal cells or cells immortalized with SV-40 and telomerase, but once they are additionally transformed by Ras they become TRAIL sensitive. This effect of Ras is associated with increased cleavage of caspase 8. To understand how this occurs we have immunoprecitate the TRAIL receptor from prostate cancer cells and human embryonic kidney cells. Only in Ras transformed cells are we able to immunoprecipitate Dr5, Dr4, FADD and caspase 8. The ability of Ras to enhance TRAIL-induced death can be blocked by inhibitors of the MEK pathway PD98059. Since this compound inhibits activation of ERK, we examined the activity of a dominant active MEK virus. We find that this MEK expressing virus when infecting immortalized cells induces can mimick the Ras effect. It makes the cells sensitive to TRAIL and increases the amount of caspase 8 bound to the receptor when TRAIL treatment is used.

To test whether this mechanism was active in tumor cells we treated TSU-PR prostate cancer cells with an inhibitor of Ras farnesylation. This chemical blocked the sensitivity of these cells to TRAIL and inhibited the ability of caspase 8 to associate with the membrane. Thus, the activation of Ras in tumor cells is key to increasing the sensitivity of these cells to TRAIL killing.

Task 3- The goal of this Task was to examine the regulation of Bid during the apoptotic process and the ability of AKT to regulate this enzyme. The ability of TRAIL to stimulate the cleavage of Bid in prostate cancer is quite low. We find that the addition of PS-341 markedly increases the ability of TRAIL to kill. This increase in ability is associated with an increase in Bid cleavage. The increase in Bid cleavage is mirrored in an increase in the release from mitochondria of cytochrome C and SMAC.

The result with Bid suggested that additional BH3 proteins might play a role in the enhancement of killing by PS-341. We have examined this process by first testing whether the Bim protein increases with PS-341 treatment. We find that Bim goes up in HC-4 colon cancer and in mouse embryo fibroblasts treated with PS-341. However, changes in the Bim level were not seen in LNCaP cells. Bim levels in these cells were high and did not change with PS-341 treatment. It is possible to hypothesize that elevated levels of Bid is necessary to inactivate Bak while Bim is needed to inactivate Bax. Additional BH3 proteins may target one of these two proteins.

Task 5- The goal of task is to determine whether normal prostate epithelial cells are sensitive to TRAIL. This work has been completed and a paper published, Nesterov, A., Ivashchenko, Y., and Kraft, A.S. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells. Oncogene 21: 1135-1140, 2002. A full set of details was submitted with the prior year's review.

Task 6- This task was to examine the role of calicitriol in enhancing TRAIL activity. As part of this project we found that PS-341, a proteasome inhibitor worked much better than calcitriol in enhancing TRAIL-induced killing. PS-341 alone, like TRAIL, was not able to kill prostate cancer cells but together they were able to kill prostate cancer cells (Fig. 1, pub 3). This combined treatment overcame the presence of bcl-xl in prostate cancer cells to induce apoptotic cell death. This is important because prostate cancer cells are known to overexpress bcl-2 family members. As part of this task we would like to take this combination into animal trials to validate it as a useful combination therapy. However, TRAIL has been shown to be toxic to human liver cells. To bypass this problem, we have established a collaboration with Human Genome Sciences Corp which has provided us with cytotoxic antibodies to the TRAIL receptor. We have shown that, like the hormone TRAIL, the ability of these antibodies to kill prostate tumor cells is enhanced by PS-341 treatment. We are now working out the dose response curve to be able to able to use these cytotoxic TRAIL antibodies and PS-341 in mice treatments.

Task 7 – This task focuses on attempting to understand the mechanism by which PS-341 enhances TRAIL activity. We find that PS-341 treatment markedly increases the cleavage of caspase 8 and Bid and causes the release of cytochrome *C* and SMAC from the mitochondria. Remarkably, PS-341 markedly increases the levels of Dr5 TRAIL receptor. These 8-fold changes in level may be a partial explanation for this enhanced killing effect. Since we find that TRAIL receptors are ubiquinated, the mechanism by which PS-341 increases the activity of TRAIL appears to be involved with the proteasome. This data is in press in Oncogene.

To understand this mechanism further we have used cells that contain a KO for Bax and Bak proteins. We find that PS-341 and TRAIL killing is inhibited by the absence of Bak and less so Bax. Therefore, Bak is needed for the enhanced killing of PS-341 and TRAIL. To further attempt to understand how Bak is activated we examined whether PS-341 treatment might increase BH3 proteins. Our PCR and western blot data show that PS-341 increases some but not all BH3 proteins. We find differences in the levels of Bim and Bik, but not PUMA. Further experiments will allow us to examine the rest of the BH3 proteins. It is our hypothesis that PS-341 treatment increases BH3 proteins that bind to Bak. Along with the increase in activated Bid (tBid) induced by the increased Dr5 receptor, we find that increased levels of BH3 proteins also have an effect on the ability of these agents to kill prostate cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- **Normal prostate epithelial cells are sensitive to TRAIL-induced apoptosis.**
- **Transformation with Ras oncogene sensitizes cells to TRAIL by increasing the amount of caspase 8 bound to FADD**
- **Ras activates Map kinase kinase to activate this caspase 8 binding**
- **Dominant active Map kinase kinase alone will sensitize cells to TRAIL-induced cell death.**
- **Prostate cancer cells are uniformily sensitized to TRAIL-induced cell death by the proteasome inhibitor PS-341, which has now been approved by the FDA for clinical practice.**
- **TRAIL plus PS-341 kills prostate cancer cells that are Bax negative or overexpress Bcl-xL.**
- **This drug combination increases cleavage of caspase 8 and Bid**
- **PS-341 and TRAIL induced killing requires the presence of Bak, but not Bax to induce cell death.**
- **PS-341 increase the level of BH3 proteins that may enhance the ability of TRAIL to kill.**

REPORTABLE OUTCOMES

Three publications have arisen from the work supported by this proposal. These works include the following:

1- Nesterov, A., Ivashchenko, Y., and Kraft, A.S. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells. Oncogene 21: 1135-1140.

2- Johnson, T., Stone, K., Nikrad, M., Yeh, T., Zong, W-X., Thompson, C.B., Nesterov, A., and Kraft, A.S. The proteasome inhibitor PS-341 overcomes TRAIL Resistance in Bax and Caspase 9-negative or bcl-xL overexpressing cells. In press Oncogene.

3- Nesterov, A., and Kraft, A.S. Oncogenic Ras sensitizes human cells to TRAIL-induced apoptosis by facilitating caspase 8 recruitment. Submitted Journ. Biol. Chem.

CONCLUSIONS

The implications of these results are important. First, we find that PS-341 treatment markedly enhances the ability of TRAIL to kill prostate cancer. PS-341 has recently received FDA human approval as Velcade. This suggests the combination of these two agents could actually reach the clinic. We have also overcome a significant problem with TRAIL. This agent has been shown to cause human liver toxicity in vitro. However cytotoxic antibodies to the receptor do not have this toxicity. We have shown that the activity of cytotoxic TRAIL antibodies is also enhanced by PS-341, suggesting that such a combination has the chance to reach the clinic.

We have been able to learn a great deal about the mechanism of this combined effect. We find that PS-341 markedly increases the number of TRAIL receptors. This increases the amount of Bid cleavage and cychrome *c* release. We find that this killing requires Bak but not Bax. In addition, PS-341 appears to increase additional BH3 proteins which lead to this phenomena. Further work will need to be done on the mechanism of action to understand how these changes enhance the killing by TRAIL in prostate cancer.

APPENDIX

1- Nesterov, A., Ivashchenko, Y., and Kraft, A.S. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells. Oncogene 21: 1135-1140.

2- Johnson, T., Stone, K., Nikrad, M., Yeh, T., Zong, W-X., Thompson, C.B., Nesterov, A., and Kraft, A.S. The proteasome inhibitor PS-341 overcomes TRAIL Resistance in Bax and Caspase 9-negative or bcl-xL overexpressing cells. In press Oncogene.

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Oncogene (2002) 21, 1135–1140

www.nature.com/onc

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells

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TRAIL is a pro-apoptotic cytokine believed to selectively kill cancer cells without harming normal ones. However, we found that in normal human prostate epithelial cells (PrEC) TRAIL is capable of inducing apoptosis as efficiently as in some tumor cell lines. At the same time, TRAIL did not cause apoptosis in several other human primary cell lines: aorta smooth muscle cells, foreskin fibroblasts, and umbilical vein endothelial cells. Compared to these primary cells, PrEC were found to contain significantly fewer TRAIL receptors DcR1 and DcR2 which are not capable of conducting the apoptotic signal. This result suggests that the unusual sensitivity of PrEC to TRAIL may result from their deficiency in anti-apoptotic decoy receptors. The protein synthesis inhibitor cycloheximide significantly enhanced TRAIL toxicity toward PrEC as measured by tetrazolium conversion but had little or no effect on other TRAIL-induced apoptotic responses. Although cycloheximide did not further accelerate the processing of caspases 3 and 8, it significantly enhanced cleavage of the caspase 3 substrate gelsolin, indicating that in PrEC a protein(s) with a short half-life may inhibit the activity of the executioner caspases toward specific substrates. As the majority of prostate cancers are derived from epithelial cells, our data suggest the possibility that TRAIL could be a useful treatment for the early stages of prostate cancer.

Oncogene (2002) **21**, 1135–1140. DOI: 10.1038/sj/onc/1205151

Keywords: TRAIL; apoptosis; primary; prostate; human

TRAIL (TNFα-Related-Apoptosis-Inducing-Ligand)/Apo-2 ligand (Wiley *et al.*, 1995; Pitti *et al.*, 1996), is a pro-apoptotic cytokine that together with three related proteins, TNFα, CD95/FasL and TWEAK/Apo3L constitute a family of ligands that transduce death signals through death domain containing receptors (Schulze-Osthoff *et al.*, 1998). TRAIL has been reported to induce apoptosis in a variety of cancer cells *in vitro*, including colon, breast, lung, kidney, CNS, blood and skin (Wiley *et al.*, 1995; Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999, 2000; Griffith *et al.*, 1999), as well as in colon and breast tumor implants in nude mice (Ashkenazi *et al.*, 1999, Walczak *et al.*, 1999). The chemotherapeutic agents 5-FU and CPT-11 can further enhance the cytotoxic effects of TRAIL (Ashkenazi *et al.*, 1999). At the same time, a large panel of primary human cells including prostate epithelial cells has been tested and reported to be TRAIL resistant (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999). Moreover, unlike TNFα and Fas ligand, whose use for cancer therapy had been hampered by their severe toxicity *in vivo* (Vassalli, 1992; Nagata, 1997), TRAIL had no toxic effects when systemically administered in rodents (Walczak *et al.*, 1999) and non-human primates (Ashkenazi *et al.*, 1999). These experimental data lead to the general belief that TRAIL can be used as a safe and specific anti-cancer agent.

Previously, we investigated the effects of TRAIL on several prostate cancer cell lines and found that their responses to TRAIL ranged from being highly sensitive (ALVA-31, DU-145, PC-3) to partially (JCA-1, TSU-Pr1) or completely (LNCaP) resistant (Nesterov *et al.*, 2001). Because TRAIL is a potential treatment for human prostate cancer, we next investigated how normal human prostate cells respond to this cytokine.

Incubation of normal human prostate epithelial cells (PrEC) with TRAIL for 6 h induced morphological changes that are characteristic of apoptosis (Figure 1a), including cell rounding and shrinkage, nuclear condensation, and membrane blebbing. To confirm that the observed changes constitute apoptosis, we tested whether TRAIL induced DNA fragmentation, a hallmark of apoptotic cell death. Figure 1b demonstrates that treatment of PrEC for 6 h with 100 ng/ml of TRAIL induced significant DNA fragmentation. To quantitate TRAIL-induced DNA fragmentation, we employed an assay that measures the release of free nucleosomes into the cytosol of apoptotic cells. Figure 1c demonstrates that TRAIL-induced DNA fragmentation reaches a maximal level at a TRAIL concentration of 60 ng/ml. Comparing these data with the published results on the sensitivity of various cancer cell lines to TRAIL-induced apoptosis (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999, 2000; Griffith *et al.*, 1999), PrEC could be classified as highly

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Received 30 January 2001; revised 19 September 2001; accepted 30 October 2001

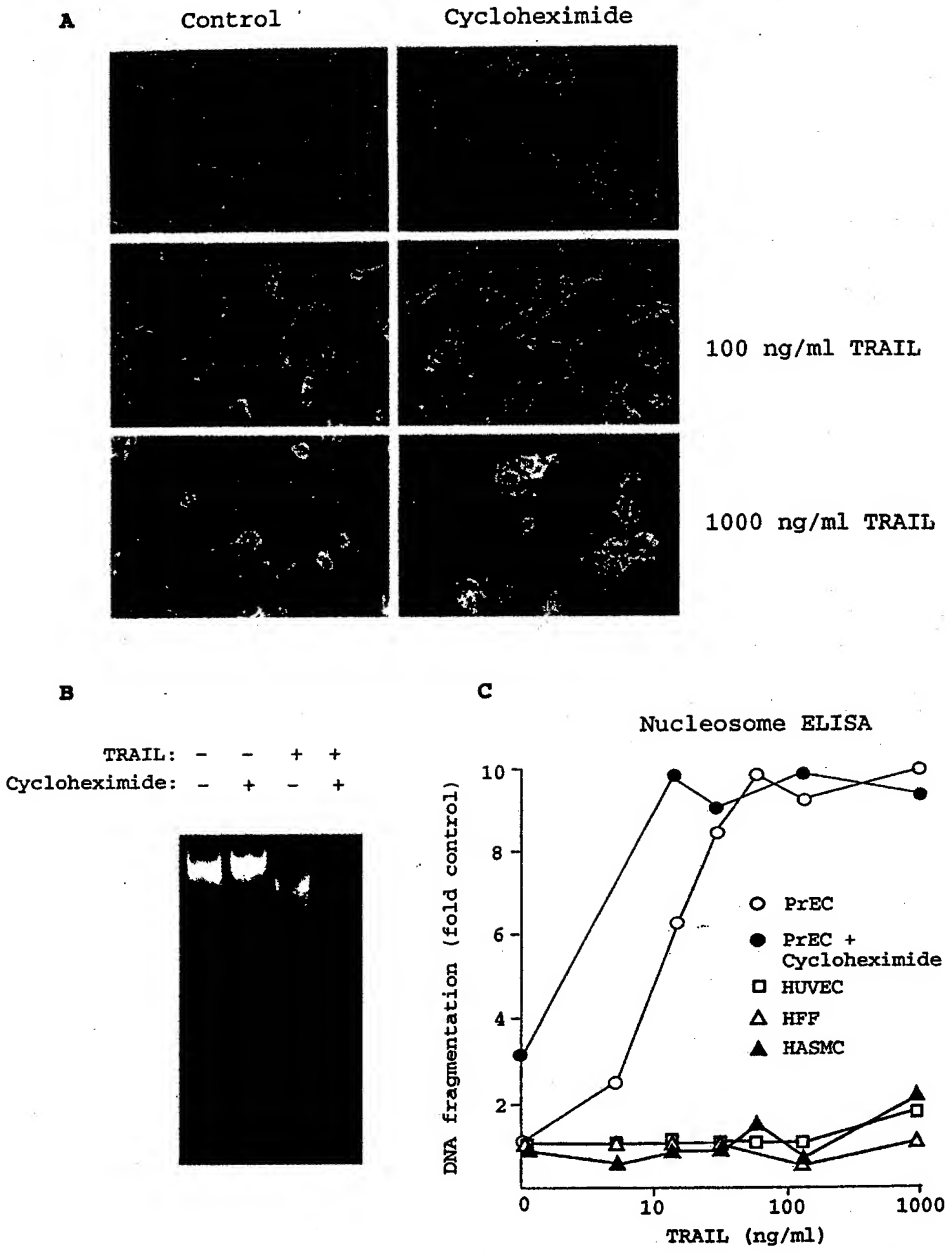

Figure 1 (**a**) Normal prostate epithelial cells (PrEC) were pre-treated overnight with 10 μM cycloheximide or left untreated (Control). Cultures were then incubated for 2 h with 100 or 1000 ng/ml of TRAIL, and visualized by light microscopy. (**b**) PrEC were pre-treated with 10 μM cycloheximide overnight or left untreated. Where indicated, cells were then incubated with 100 ng/ml of TRAIL for 6 h and DNA fragmentation was monitored by electrophoresis in a 2% agarose gel. (**c**) PrEC, human foreskin fibroblasts (HFF), human umbilical vein endothelial cells (HUVEC), and human aorta smooth muscle cells (HASMC) were incubated for 6 h with TRAIL at concentrations increasing from 7–1000 ng/ml. DNA fragmentation was quantitated by measuring the relative amounts of DNA-histone complexes released into cytoplasm using Cell Death Detection ELISA^{plus} Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). In one case PrEC were pre-treated overnight with 10 μM cycloheximide prior to the experiment. All primary huamn cells were purchased from Clonetics (Biowhittaker Products, Walkersville, MD, USA) and maintained as outlined by the supplier. The expression and purification of recombinant TRAIL was described in detail elsewhere (Nesterov *et al*., 2001). Briefly, soluble TRAIL was generated by expression of amino acids 114–281 of human TRAIL fused with amino-terminal $(His)_6$ tag in yeast *Pichia pastoris*. The secreted protein was purified to homogeneity from the yeast supernatant by Ni-chelate chromatography

TRAIL-sensitive. Since this finding contradicted the general concept that TRAIL toxicity is limited to cancerous cells, we tested whether our preparation of TRAIL possessed an aberrant toxicity toward normal cells. It was found that our TRAIL protein did not cause apoptosis in several other primary cells: human foreskin fibroblasts (HFF), human umbilical cord endothelial cells (HUVEC), and human aorta smooth muscle cells (HASMC) (Figure 1c). Consistent with previously published data (Ashkenazi *et al*., 1999; Walczak *et al*.,

1999), systemic administration of our TRAIL to athymic mice for up to 10 days (100–500 μg/animal) did not produce any toxic effects (data not shown). Compared with two prostate cancer cell lines, ALVA-31 and LNCaP cells, PrEC displayed an intermediate sensitivity to TRAIL (Figure 2a,b). Thus, contrary to the concept that malignant cells are more sensitive to this protein than normal ones (Wiley *et al.*, 1995; Leverkus *et al.*, 2000), it appears that cells may either decrease or increase their sensitivity to this agent upon transformation.

The finding that, unlike some other human primary cells, PrEC appear to be unusually sensitive to TRAIL raised the question of whether these cells contain an abnormally high number of TRAIL receptors. Quantitative PCR data (Figure 2c) demonstrated that the amount of mRNA for TRAIL receptors DR4 and DR5 in PrEC was comparable to that found in TRAIL-resistant HUVEC and HASMC. However, the proportional amount of mRNA for TRAIL receptors DcR1 and DcR2, which bind TRAIL but do not transduce a death signal, was significantly lower in PrEC and TRAIL-sensitive prostate cancer cells ALVA-31 than in other primary cells and TRAIL-resistant prostate cancer cells LNCap. Thus, one possible explanation for the unusual sensitivity of PrEC to TRAIL is that these cells have fewer 'decoy' receptors.

Since our data contradicted the previous report (Ashkenazi *et al.*, 1999) where PrEC were found to be TRAIL-resistant, we performed a series of experiments to confirm the specificity of our observation. First, to

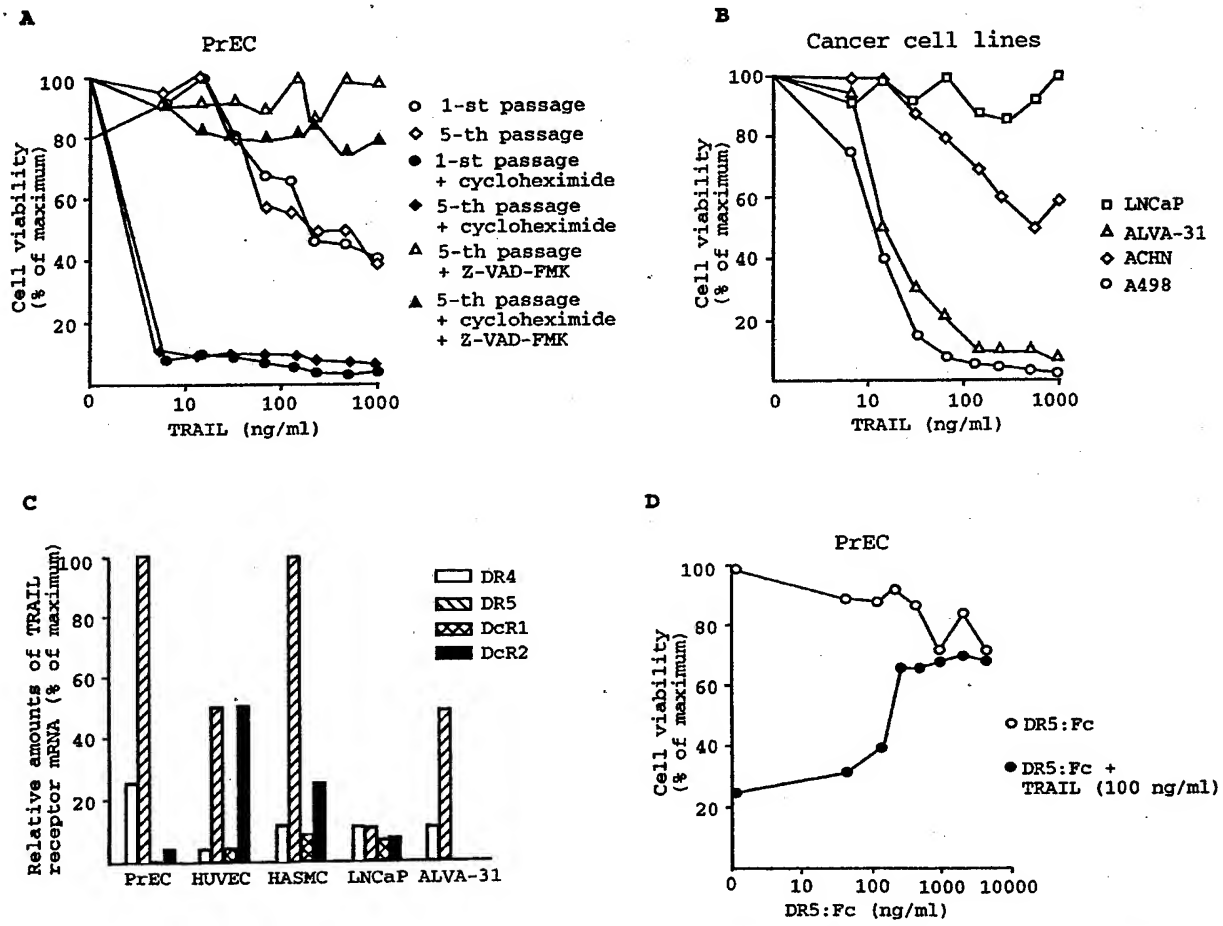

Figure 2 (a) Normal human prostate epithelial cells on 1st and 5th passages were pre-treated with 10 μM cycloheximide overnight or left untreated. Cells were then incubated for 24 h with TRAIL alone at concentrations increasing from 7–1000 ng/ml or in combination with 40 μM of Z-VAD-FMK (Enzyme Systems Products, Livermore, CA, USA). Cell viability was determined spectrophotometrically using an MTS tetrazolium based assay (Promega, Madison, WI, USA). Absorbance was measured at 490 nm and data from quadruplicate determinations was plotted as per cent of maximal signal. (b) Human prostate cancer cell lines LNCaP and ALVA-31, and renal cancer cell lines ACHN and A498, were incubated for 24 h with TRAIL at concentrations increasing from 7–1000 ng/ml. Cell viability was determined as described in the legend to a. (c) Relative amounts of mRNA for pro-apoptotic TRAIL receptors DR4 and DR5 and 'decoy' receptors DcR1 and DcR2 in primary cells and prostate cancer cell lines were determined by quantitative PCR using β-actin as a standard. RNA was extracted from cells using TRIzol reagent (GibcoBRL, Rockville, MD, USA). Real time PCR was performed using iCycler (BioRad, Hercules, CA, USA). (d) PrEC were treated for 36 h with soluble extracellular domain of TRAIL receptor DR5 (DR5:Fc) (Alexis, San Diego, CA, USA) alone at concentrations increasing from 64 to 8000 ng/ml or in the presence of 100 ng/ml of TRAIL. Cell viability was determined as described in the legend to a

rule out the possibility that TRAIL produced in our laboratory is significantly more potent than that used by others, we tested its efficacy on two renal cancer cell lines, ACHN and A498 (Figure 2b), which were also examined by Ashkenazi *et al.*, 1999. The results we obtained using our TRAIL preparation were comparable to the published data: 100 ng/ml of TRAIL reduced cell viability approximately 2–5-fold for ACHN and A498 cells respectively. Second, using pan-caspase inhibitor Z-VAD-FMK, we confirmed that the effect of our TRAIL on PrEC was mediated by caspases (Figure 2a), suggesting that our TRAIL was activating the normal apoptotic pathway. Third, we found that the soluble extracellular domain of TRAIL receptor DR5 (DR5:Fc) is capable of inhibiting TRAIL-induced apoptosis in PrEC (Figure 2d), indicating that the effect of TRAIL is receptor-mediated and does not result from a toxic contaminant of our preparation.

Since the response of cells to various apoptotic stimuli may change with aging (Warner *et al.*, 1997) we also tested whether the conflicting results could arise from the differences in the age of cell cultures. In our experiments PrEC did not change their growth characteristics during the first five passages, with senescent cells beginning to appear after passage six. The data presented in Figure 2a demonstrate that young (1st passage) and aged (5th passage) cultures are equally sensitive to TRAIL.

It is possible that the discrepancies between our results and those previously published may arise from the assays chosen to quantitate apoptosis. For example, in the apoptosis-specific DNA fragmentation assay, internucleosomal DNA fragmentation reached a maximum at a TRAIL concentration of 60 ng/ml (Figure 1c). However, in the tetrazolium conversion assay, a technique that measures general cell viability (Cory *et al.*, 1991), the maximal effect could not be achieved even at 1000 ng/ml of TRAIL (Figure 2a). In the report by Ashkenazi *et al.*, 1999, PrEC were found to be TRAIL-insensitive, judging by propidium iodide staining. A possible drawback to the use of vital dyes, such as propidium iodide, is that cells undergoing apoptosis may retain cell membrane integrity and appear alive until late in the apoptotic program when secondary necrosis begins (Loo and Rillema, 1998). For example, in our experiments, staining of PrEC with vital dye, trypan blue, did not detect a significant number of dead cells if they were treated with TRAIL for less than 24 h (data not shown).

The pro-apoptotic effects of TRAIL can often be enhanced by the inhibition of protein synthesis (Griffith *et al.*, 1998; Kreuz *et al.*, 2001; Wajant *et al.*, 2000), suggesting that proteins with a short half-life protect cells from TRAIL-induced death. Therefore, we tested whether the inhibition of protein synthesis by cycloheximide affects TRAIL-induced apoptosis in PrEC. As demonstrated by Figure 1, cycloheximide had little or no effect on TRAIL-induced morphological changes and only moderately increased sensitivity of cells to this agent in DNA fragmentation assays. However, as measured by the tetrazolium conversion assay (Figure 2a) which assesses cell viability based on the respiratory function of mitochondria (Cory *et al.*, 1991), the inhibition of protein synthesis dramatically enhanced TRAIL toxicity. These results suggest that some, but not all TRAIL-induced apoptotic responses in PrEC are partially inhibited by short-lived anti-apoptotic proteins. As demonstrated by Figure 3, processing of the initiator caspase 8, the effector caspase 3, and one of caspase 3 substrates, the inhibitory subunit of DNA fragmentation factor (DFF45/ICAD) (Cryns and Yuan, 1998), were induced by TRAIL alone as efficiently as when TRAIL was combined with cycloheximide. In contrast, cyclohex-

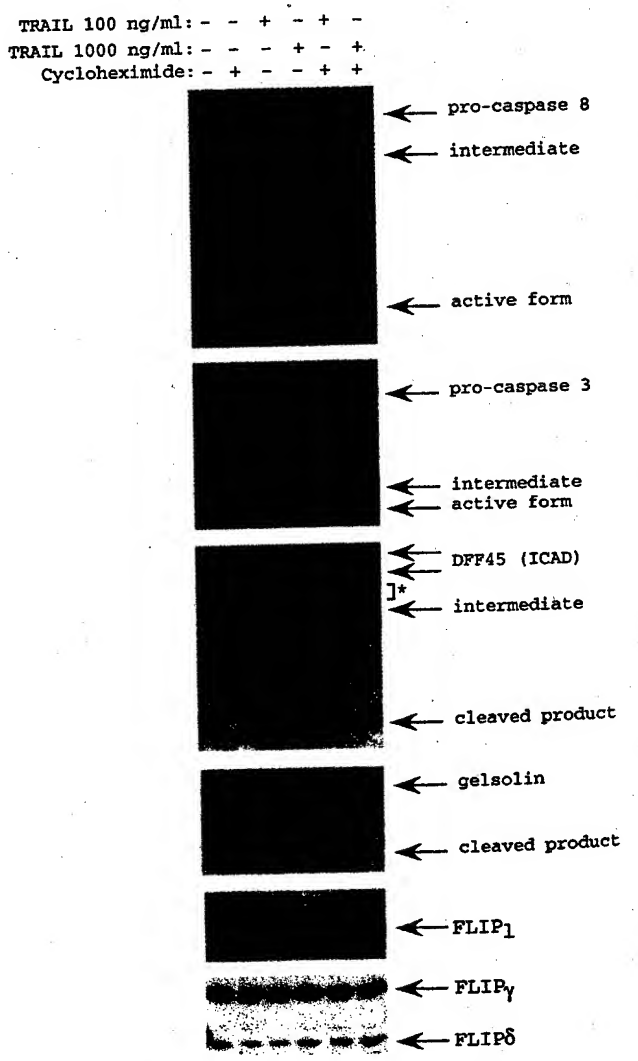

Figure 3 PrEC were pre-treated overnight with 10 μM cycloheximide or left untreated. Cultures were then incubated for 6 h with 100 or 1000 ng/ml of TRAIL alone or in combination with cycloheximide. Cell lysates were electrophoresed and the same Western blot was consecutively probed with antibodies specific to caspase 8 (Upstate Biotechnology, Lake Placid, NY, USA), caspase 3 (Transduction Laboratories, Lexington, KY, USA), DFF45 (Zymed Laboratories, So. San Francisco, CA, USA), gelsolin (Sigma, St. Louis, MO, USA), FLIP$_L$ (ABR, Golden, CO, USA) and FLIP γ/δ (Calbiochem, San Diego, CA, USA)

imide significantly enhanced processing of gelsolin, a prominent caspase 3 substrate, implicated in mediating apoptotic cytoskeletal changes (Kothakota *et al.*, 1997). This result indicates that in PrEC a short-lived anti-apoptotic proteins(s) may inhibit the activity of the effector caspases toward specific substrates.

It has been recently reported that the inhibition of protein synthesis significantly enhances TRAIL-induced apoptosis in primary human keratinocytes (Kothakota *et al.*, 1997). In keratinocytes, cycloheximide down-regulated FLIPs, proteins implicated in caspase 8 inhibition (Tschopp *et al.*, 1998) and accelerated TRAIL-induced caspase 8 processing. However, in PrEC cycloheximide did not affect either the level of FLIPs or the rate of caspase 8 processing (Figure 3). This result indicates that the biologic effects of cycloheximide may be cell type specific.

Together with the recent data on TRAIL cytotoxicity toward primary human keratinocytes (Leverkus *et al.*, 2000) and hepatocytes (Jo *et al.*, 2000) our results challenge the concept of the cytotoxic effects of TRAIL being limited to transformed cells. While this paper was under review, Lawrence *et al.*, 2001 reported that different preparations of TRAIL may have different effects on primary cells. Based on the observation that recombinant TRAIL containing a hexahistidine tag induced apoptosis in primary human hepatocytes, whereas TRAIL without this tag did not, the authors concluded that the toxicity of TRAIL toward normal cells depended on the presence of hexahistidine. However, the data on primary human keratinocytes demonstrated that recombinant TRAIL which lacks a hexahistidine sequence may also trigger apoptosis in certain normal cells (Lawrence *et al.*, 2001). Since we were not able to obtain TRAIL preparations used in the above referenced studies, we cannot preclude that the toxicity of our TRAIL toward prostate epithelial cells resulted from the presence of a hexahistidine tag. However, the effect of our TRAIL on PrEC did not appear to result from non-specific toxicity because: (1) it was dependent on caspase activity; (2) it could be inhibited by the extracellular domain of TRAIL receptor; and (3) it was accompanied by proteolytic events typical of receptor-mediated apoptosis. The observation that PrEC were unusually sensitive to TRAIL may have important implications for TRAIL-based prostate cancer therapy. As the majority of prostate cancers are derived from the epithelial cells (Stamey and McNeal, 1992), it is possible that TRAIL could be used for the treatment of developing premalignant lesions or early stages of prostate cancer. At the same time, our data raise concerns about TRAIL safety when this agent is used as a systemic drug for cancer therapy. It cannot be ruled out that some other human tissues may also be sensitive to this agent; thus, more extensive studies are needed to evaluate TRAIL sensitivity of multiple other primary cell lines before TRAIL is used for human treatment. A better understanding of the mechanisms involved in the inhibition of receptor-mediated cell death may also be beneficial for deciphering control of TRAIL-induced apoptosis in normal and malignant cells.

Acknowledgments

We acknowledge Fr Elke Kessler for her help with the primary cultures of HUVECs, Human aortic smooth muscle cells and experiments performed with these cells; Dr Pearly Lee with Real Time PCR experiments. This work was supported by NIH grant CA 78631 to AS Kraft.

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Oncogene (2003) 00, 1–11

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ORIGINAL ARTICLE

The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells

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We demonstrate that PS-341, a small molecule inhibitor of the proteasome, markedly sensitizes resistant prostate, colon, and bladder cancer cells to TNF-like apoptosis-inducing ligand (TRAIL)-induced apoptosis irrespective of Bcl-xL overexpression. PS-341 treatment by itself does not affect the levels of Bax, Bak, caspases 3 and 8, c-Flip or FADD, but elevates levels of TRAIL receptors DR4 and DR5. This increase in receptor protein levels is associated with the ubiquitination of the DR5 protein. When PS-341 is combined with TRAIL, the levels of activated caspase 8 and cleaved Bid are substantially increased. In Bax-negative TRAIL-resistant HC-4 colon cancer cells, the combination of PS-341 and TRAIL overcomes the block to activation of the mitochondrial pathway and causes SMAC and cytochrome *c* release followed by apoptosis. Similarly, murine embryonic fibroblasts lacking Bax undergo apoptosis when exposed to the combination of PS-341 and TRAIL; however, fibroblasts lacking Bak are significantly resistant. Taken together, these findings indicate that PS-341 enhances TRAIL-induced apoptosis by increasing the cleavage of caspase 8, causing Bak-dependent release of mitochondrial proapoptotic proteins.
Oncogene (2003) **0**, 000–000. doi:10.1038/sj.onc.12466

Keywords: PS-341; TRAIL; Bax; Bcl-2; apoptosis

Introduction

TNF-like apoptosis-inducing ligand (TRAIL) and PS-341 are two novel anticancer agents with differing mechanisms of action. TRAIL induces apoptosis by first binding to two closely related receptors DR4 and DR5, causing the formation of a death-inducing signaling complex (DISC), which includes the receptors, the adaptor protein FADD, and caspase 8 (Medema *et al.*, 1997). Autoactivated caspase 8 is capable of cleaving procaspase 3 directly or digesting Bid to its active form (tBid), which leads to the release of cytochrome *c* from the mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998; Gross *et al.*, 1999). PS-341 is a small molecule inhibitor of the proteasome that induces apoptotic cell death (Teicher *et al.*, 1999). Although treatment with this compound causes marked increases in a large number of cellular proteins (Adams *et al.*, 1999), including c-*myc* and the cyclin-dependent protein kinase inhibitor $p21^{WAF1}$, it is not clear how this agent actually induces apoptosis. Tumor cells that do not express p53 or p21 still die when incubated with this agent (An *et al.*, 2000). PS-341 inhibits the activation of NF-κB (Sunwoo *et al.*, 2001). NF-κB plays a role in controlling the levels of inhibitor of apoptosis proteins, IAPs (Wang *et al.*, 1998); however, the regulation of NF-κB activity has not been directly linked to a specific mechanism by which PS-341 induces apoptosis. Through an unknown mechanism, PS-341 treatment of tumor cells overcomes drug resistance, and can enhance the killing activity of both dexamethasone and chemotherapeutic agents (Cusack *et al.*, 2001; Hideshima *et al.*, 2001). This effect would appear additive and not synergistic.

Despite widespread expression of TRAIL receptors, many tumor cell lines are either partially or completely resistant to apoptotic cell death induced by this agent (Hao *et al.*, 2001; Pawlowski *et al.*, 2001). Resistance results from a number of factors including the absence of caspase 8 (Grotzer *et al.*, 2000), increased AKT activity resulting from deletion of the PTEN phosphatase (Nesterov *et al.*, 2001), or the absence of Bax (Deng *et al.*, 2002). In addition, overexpression of Bcl-2 family members blocks TRAIL-induced apoptosis (Munshi *et al.*, 2001; Roklin *et al.*, 2001). The observation that the majority of TRAIL-resistant cell lines can be made TRAIL sensitive by treatment with protein synthesis inhibitors (Rieger *et al.*, 1998; Wajant *et al.*, 2000) implies that additional mechanisms regulating TRAIL resistance may exist.

Our results indicate that PS-341 and TRAIL synergize to kill TRAIL-insensitive tumor cell lines, including those overexpressing Bcl-xL or deficient in Bax, but is much less effective in cells lacking Bak. The effects of PS-341 appear to be mediated by its ability to enhance TRAIL-stimulated cleavage of caspase 8, which may be

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This work was supported by Department of Defense Grant DAMD17-99-1-9516 to ASK.

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correlated with increased expression of TRAIL receptors DR4 and DR5. Our results suggest that this combination of agents may be useful therapeutically in killing tumor cells with multiple abnormalities in the apoptotic pathway.

Materials and methods

Cell lines and reagents

The human prostatic carcinoma cell line LNCaP and bladder JCA-1 cells were cultured in RPMI 1640 (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS, 100 U/ml penicillin/100 μg/ml streptomycin. HCT-116, HC-4, and murine embryonic fibroblasts (MEF) were cultured in DMEM (Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS, 100 U/ml penicillin/100 μg/ml streptomycin. MEF were frozen at passage 2 and used at the first passage after thawing. Antibodies were obtained from the following sources: DR4 and DR5 antibodies, Alexis (San Diego, CA, USA); ubiquitin antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); Bcl-2, caspase 8, Upstate Biotechnology (Lake Placid, NY, USA); Poly ADP ribose polymerase (PARP), Trevigen (Gaithersburg, MD, USA); $P21^{waf1}$ and FADD antibodies, BD Transduction Laboratories (San Jose, CA, USA); SMAC and myc antibodies, University of Colorado Health Science Center Monoclonal Antibody Core Facility (Denver, CO, USA); FLAG agarose, FLAG and HA antibodies, Sigma Chemical Co. PS-341 was a gift of Millennium, Inc. (Cambridge, MA, USA). The FLAG-DR5 L334N expression vector was constructed as follows: the sequence corresponding to residues 56–411 of mature human DR5 was amplified from a template supplied by Genentech. This sequence was inserted into the expression vector pFLAG-CMV1 (Sigma) to provide an N-terminal signal peptide from preprotrypsin and the FLAG epitope. The L334N mutation in the death domain was then made with the QuickChange kit (Stratagene), and the entire epitope-bearing sequence transferred to the episomal vector pCEP4 (Invitrogen). The integrity of the construct was verified by DNA sequencing.

Recombinant human TRAIL

TRAIL cDNA was subcloned into the pET-15b vector (Novagen, Madison, WI, USA) and used to transform *Escherichia coli* strain BL21 (Promega, Madison, WI, USA). Recombinant human TRAIL was purified on a Ni-NTA agarose column (Qiagen, Valencia, CA, USA) after cell lysis in 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 1 mM PMSF, 5 mM 2-mercaptoethanol, and 2.5% Triton X-100.

Cell viability assays

To measure cell viability, LNCaP and LNCaP-Bcl-xL cells were plated in 96-well plates at 10 000 cells/well and incubated overnight. The cells were then treated with varying amounts of DMSO, PS-341, TRAIL, or the combination. After a 16 h incubation at 37°C, MTS substrates (Cell TITER 96 Aqueous Assay, Promega, Madison, WI, USA) were added according to protocol, and absorbance at 490 nm was measured with a *v*Max Kinetic Plate Reader (Molecular Devices, Sunnyvale, CA, USA). For assays employing 4′,6-diamido-2-phenylindole (DAPI), cells were plated in six-well plates 24 h prior to treatments. At the end of the treatment period, cells were washed, trypsinized, centrifuged, and resuspended in 0.05 ml complete medium containing 1 μg/ml DAPI. All components of the harvesting step (culture medium, washes, and trypsinized cells) were combined before centrifugation to ensure recovery of unattached cells. Cells were examined and nuclei with apoptotic morphology were counted using a Zeiss Axiophot fluorescence microscope with an appropriate filter at $\times$400 magnification. DAPI experiments were performed in duplicate and a minimum of 300 cells per sample were scored.

Western blot analysis

Cell lines were plated in six-well plates and treated with appropriate amounts of DMSO as a control, PS-341, or TRAIL under a variety of conditions. The cells were then lysed in SDS–PAGE sample buffer (15% glycerol, 0.125 M Tris-HCl, pH 6.8, 5 mM EDTA, 2% SDS, 0.1% bromophenol blue, 1% 2-mercaptoethanol), and incubated for 10 min at room temperature. The lysate was then boiled for 5 min and frozen at −80°C. Western blots were carried out as described previously (Nesterov *et al*., 2001).

Quantitative RT–PCR

Quantitative RT–PCR assays were performed using the ABI 5700 real-time system with SYBR-green fluorescence, as described in detail (Drabkin *et al*., 2002). Briefly, cDNA was synthesized from 2.5 μg of total RNA isolated by the method of Chomczynski and Sacchi (1987) with Superscript II (Invitrogen Life Technologies, Carlsbad, CA, USA) using random primers and conditions recommended by the manufacturer. For quantitative RT–PCR, 20 μl reactions were utilized, which included 1 μl cDNA, 1.6 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphates, 0.1 μM primers, and 0.1 μl AmpliTaq Gold (Applied Biosystems, Wellesley, MA, USA). Following an initial 95°C 10 min incubation to activate the polymerase, 40 cycles of a two-stage PCR were performed consisting of 95°C $\times$15 s and 60°C $\times$1 min. Duplicate reactions were performed that invariably agreed within 1/2 cycle. RNA used for cDNA synthesis was measured both by optical density and gel electrophoresis followed by densitometric scanning. PCR products were verified by gel analysis. Absence of contamination was verified by the use of no template controls and mock cDNA synthesis reactions without reverse transcriptase. Data were analysed with GeneAmp 5700 SDS software. Primers were DR4F: ATGGCGCCACCAC-CAGCTAGAG; DR4R: GGAGGTAGGGAGCGCTCCTC-GG; DR5F: ATCACCCAACAAGACCTAGC; DR5R: CA-CCTGGTGCAGCGCAAGCAG. Semiquantitative PCR for DR5 was performed with cDNAs prepared as above. Primers were DR5F: GGGAAGAAGATTCTCCTGAGATGT; DR-5R: ACATTGTCCTCAGCCCCAGGTCG, which span the splice junction (Screaton *et al*., 1997).

Isolation of TRAIL receptors DR4 and DR5

Cells were detached from plates by incubation with Hank's Basic Salt Solution supplemented with 2 mM EDTA and 2 mM EGTA. The isolation was performed in two ways. (1) For isolation of total cellular receptors, cells were pelleted, washed in PBS, and lysed in TGH buffer (50 mM HEPES pH 7.2, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.25 mM phenylmethylsulfonylfluoride, and protease inhibitors) for 15 min on ice. Lysates were centrifuged for 30 min at 100 000 *g*. Histidine-tagged TRAIL prepared as described above was added at 1 μg/ml to the supernatants, which were incubated for 20 min on ice. Anti-histidine antibody coupled to agarose beads (Sigma) was added to the supernatants, which were then incubated with rotation for 2 h to overnight at 4°C. Beads were washed

thoroughly with TGH buffer and eluted twice with 100 mM glycine (pH 2.3) for 10 min on ice. Following addition of concentrated SDS–PAGE sample buffer, the eluates were neutralized with 1 N NaOH and boiled for 5 min prior to electrophoresis. (2) For isolation of cell surface receptors and associated DISC components, following detachment from culture dishes, cells were pelleted and resuspended in culture medium set aside from the individual treatments. Histidine-tagged TRAIL was added to 1 μg/ml and the cells were incubated at 37° for 20 min with agitation. After assessment of viability by trypan blue exclusion, cells were pelleted, washed, lysed, and centrifuged as described above. The remainder of the procedure was as described except that TRAIL was not added to the cell lysates. Before the addition of anti-histidine agarose, the protein content of the lysates was determined and volumes adjusted to equalize protein content.

Cytosolic SMAC levels

Cytosolic extracts from HC-4, HCT-116, and LNCaP cells were prepared by the procedure originally described by Bossy-Wetzel *et al.* (1998) as modified by Nesterov *et al.* (2001). Briefly, 10-cm plates of cells near-confluence were scraped directly into cell culture medium and pelleted for 5 min at 200 *g*. Cells were washed once in 1 ml phosphate-buffered saline (PBS) and resuspended in 0.35 ml HT buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES–KOH pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) by vortexing. After 45 min on ice, cells were revortexed and passed 10 times through a 25 gauge needle. Following 10 min centrifugation at 200 *g*, the supernatant was cleared by centrifugation for 30 min at 100 000 *g* and analysed for SMAC protein by Western blotting.

Nucleosome assay

LNCaP cells were grown to confluency in 10-cm plates. They were then treated with DMSO, 1 μM PS-341, 100 ng/ml TRAIL, or a combination of PS-341 and TRAIL. The following day, the cells were collected via trypsinization, pelleted and lysed according to the Nucleosome ELISA kit (Oncogene Research Products, Boston, MA, USA). The subsequent assay was carried out as directed.

Crystal violet staining

HCT-116 and HC-4 cells were plated in 24-well plates and grown to confluency. They were then treated with varying concentrations of PS-341 and/or TRAIL. Medium was gently aspirated and cells were washed in 1 × PBS. Crystal violet stain was prepared with 0.1% crystal violet in ddH_2O and added to wells until stain covered the bottom of the wells. The plates were then incubated at room temperature for 10 min. The stain was then gently aspirated and the wells were washed three times with 1 × PBS.

X-Gal staining of caspase 8-transfected cells

HCT-116 and HC-4 cells were plated in six-well plates and grown to approximately 50% confluency. They were then cotransfected with 0.2 μg LacZ plasmid (Invitrogen) and 0.2 μg Fkp-caspase 8 (180) plasmid (a gift of Dr David Baltimore, California Institute of Technology, Pasadena, CA, USA). The cells were incubated for 24 h and stained with X-Gal. The experiment was performed in triplicate and repeated twice.

Results

To determine whether PS-341 enhances the ability of TRAIL to induce cell death, LNCaP human prostate cancer cells were incubated with either TRAIL, PS-341, or the combination for 24 h and a cell viability (MTS) assay was carried out. LNCaP cells showed no change in viability after treatment with either PS-341 or TRAIL, but the combination of both led to significant decrease in viability (Figure 1a). Using the analysis of Chou and Talalay (1983), the combination of these two agents was found to be highly synergistic (data not shown). To establish that the decrease in viability was caused by induction of programmed cell death (apoptosis), an ELISA assay was performed, which measures free cleaved nucleosomes generated during apoptosis. Only addition of both PS-341 and TRAIL, but not TRAIL or PS-341 alone, induced increases in free nucleosomes (Figure 1b). Similar results were obtained with multiple prostate cancer cell lines, including PC-3 and DU-145 cells (data not shown).

To evaluate the possibility that the failure of PS-341 alone to kill LNCaP cells reflected an inability of the drug to penetrate these cells, levels of specific cellular proteins after treatment were measured. Since PS-341 inhibits proteasome degradation, we expected to see an increase in ubiquinated proteins. An 18 h treatment of LNCaP cells with PS-341 caused marked increases in total ubiquinated proteins, as well as two proteins normally degraded by the proteasome system, $p21^{WAF-1}$ and c-*myc* (Figure 1c), whereas there was no change in the levels of PARP protein. It is possible that the increase in $p21^{WAF-1}$ seen when incubation with PS-341 is compared to the combined treatment may come from an increased ability to extract this protein in cells undergoing apoptosis. Only the combination of PS-341 plus TRAIL, but not PS-341 alone, induced the cleavage of PARP (Figure 1c), consistent with the ability of these agents when combined to induce apoptosis.

Overexpression of Bcl-2 family members blocks TRAIL-induced cell death (Munshi *et al.*, 2001; Roklin *et al.*, 2001), and release of SMAC and cytochrome *c* from mitochondria. To test whether PS-341 could overcome this block, LNCaP prostate cancer cells overexpressing the Bcl-xL protein were incubated with PS-341, TRAIL, or the combination (Figure 2a). Approximately threefold overexpression of Bcl-xL compared to parental LNCaP cells was verified (data not shown). MTS assay results demonstrated that whereas each agent alone was unable to decrease cell viability, the combination was able to overcome Bcl-xL overexpression, leading to marked decrease in cell viability. Parallel experiments demonstrated that only the combination of PS-341 and TRAIL resulted in the appearance of SMAC protein in cytosolic extracts of these cells (data not shown).

TRAIL-induced cleavage of Bid to tBid enables the translocation of Bax protein into mitochondria followed by the release of cytochrome *c* and induction of apoptosis (Wei *et al.*, 2001). To investigate whether TRAIL and PS-341 could induce the death of cells that

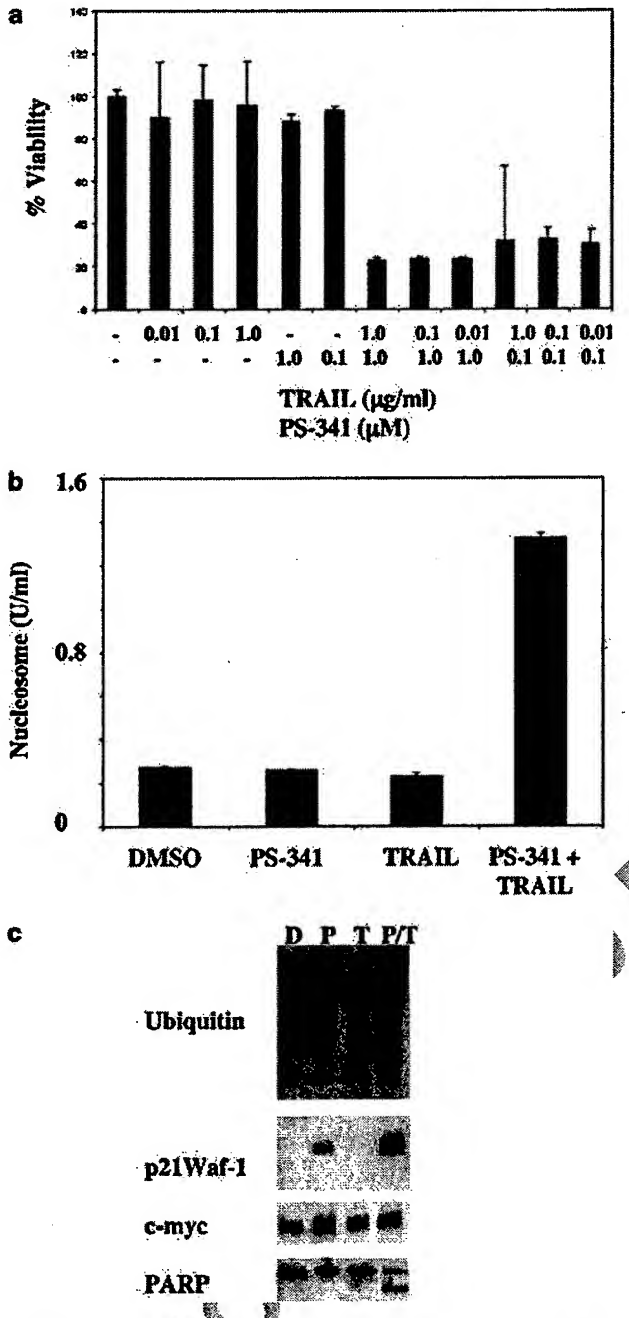

Figure 1 PS-341 treatment markedly enhances TRAIL-induced apoptosis. (**a**) Cell viability assay of cell growth. LNCaP cells were treated with PS-341 or TRAIL in varying amounts overnight and the MTS assay was performed as described in Materials and methods. The s.d. of triplicate determinations is shown. (**b**) Nucleosome ELISA assay of treated LNCaP cells. LNCaP cells were treated overnight with vehicle, PS-341 (1 μM), TRAIL (0.1 μg/ml), or the combination of these two agents. The s.d. of triplicate determinations is shown. (**c**) The effect of PS-341, TRAIL, or both agents on the levels of specific cellular proteins. LNCaP cells were treated as in Figure 1b (D, DMSO; T, TRAIL; P, PS-341, and P/T, PS-341 and TRAIL). Extracts were run on a 10% SDS : PAGE gel, transferred to Immobilon, and Western blotted with antibodies to ubiquitin, $p21^{waf-1}$, c-Myc, and PARP

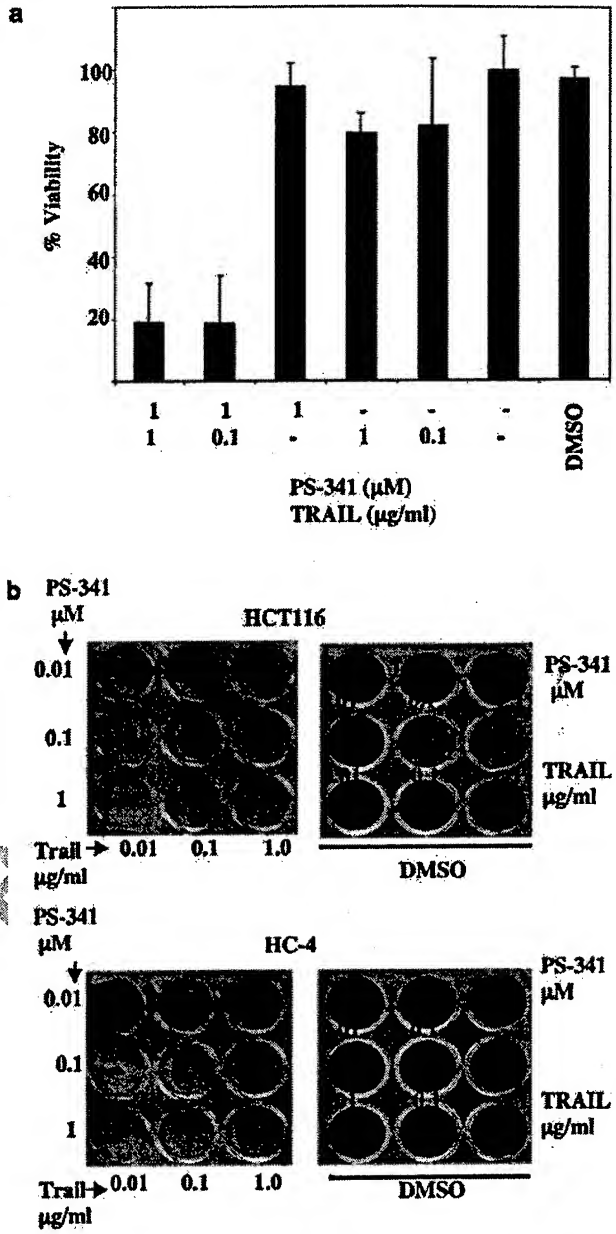

Figure 2 The combination of TRAIL and PS-341 overcomes Bcl-xL overexpression and the absence of Bax to induce cell death. (**a**) LNCaP-Bcl-xL cells were treated overnight with either PS-341 or TRAIL or both agents. An MTS assay was performed. The s.d. of measurements made in triplicate is shown. (**b**) Treatment of HCT-116 and HC-4 cells with varying concentrations of PS-341 (P) or TRAIL (T) or both agents. Cells were treated overnight and then stained with crystal violet as described in Materials and methods. The first block of tissue culture wells of each cell type was treated with both agents, including increasing amounts of PS-341 (down arrow) plus increasing levels of TRAIL (right arrow). The second block of wells from each cell line was treated with these agents individually, increasing PS-341 concentrations (top row), TRAIL concentrations (middle row), or DMSO, the vehicle (bottom row)

are missing Bax, two colon and one prostate cancer cell line were examined. The parental colon cancer cell line HCT-116 expresses Bax protein and is highly sensitive to TRAIL-induced death, while the variant HC-4 cell line

derived from HCT-116 contains a frame-shift mutation yielding cells that do not contain Bax protein and are resistant to TRAIL-induced cell death (Rampini *et al.*, 1997). Using crystal violet staining as a means of detecting live cells, we incubated HC-4 and HCT-116 cells with these agents alone or in combination. The results demonstrated (Figure 2b) that the addition of either 0.1 or 1 μM PS-341 had no or minimal effect on these cells, whereas the addition of PS-341 enabled TRAIL to kill the HC-4 cells, and lowered the concentration of TRAIL necessary to kill HCT-116 cells. Like HC-4 cells, DU-145 human prostate cancer cells also contain a frame-shift mutation in Bax. These cells also demonstrated a combined response of TRAIL and PS-341 (data not shown).

To test whether PS-341 enhances TRAIL-mediated apoptosis by upregulating proteins in the TRAIL pathway, we examined the levels of specific proteins in the pathway after treatment with each agent alone or together. In LNCaP cells, PS-341 did not induce changes in the levels of FADD, Bak, or Bax (Figure 3a); or caspases 3, 8 or XIAP (data not shown). Although levels of caspase 8 and FADD were unchanged after PS-341 treatment, we found that overnight treatment of LNCaP cells with PS-341 and TRAIL resulted in increased cleavage of caspase 8, as evidenced by elevated levels of 41, 43, and 18 kDa cleavage products (Figure 3b). This increase in caspase 8 cleavage was associated with a decrease in levels of Bid (Figure 3b), a caspase 8 substrate, and activation of the effector caspases 3 and 7 (data not shown). Quantitation of these Western blots using scanning densitometry demonstrated a 50% increase in the levels of the 18 kD caspase 8 fragment with the combined treatment compared to treatment with TRAIL alone. Similar results were obtained with the JCA-1 human bladder cancer cell line (Figure 3c). The figure shows that in the presence of PS-341 the extent of caspase 8 and Bid cleavage is directly related to the dose of TRAIL applied.

Recent evidence suggests that c-FLIP plays an important role in TRAIL sensitivity, and that down-regulation of functional c-FLIP in response to doxorubicin treatment (Kelly *et al.*, 2002), cell differentiation (Hietakangas *et al.*, 2003), or phosphorylation (Higuchi *et al.*, 2003) increases sensitivity to TRAIL-mediated apoptosis. In addition, FLIP is subject to ubiquitination and proteasomal degradation (Kim *et al.*, 2002). To determine the role of FLIP in PS-341 action, we examined levels of c-FLIP-L and c-FLIP-S in HC-4 and LNCaP cells using an antibody that recognizes both forms of c-FLIP (Scaffidi *et al.*, 1999). The results are shown in Figure 3d. In both cell lines, c-$FLIP_L$, and c-$FLIP_S$ appear upregulated in response to PS-341. In addition, the levels of the p43 cleavage product appear equivalent in samples treated with TRAIL or the

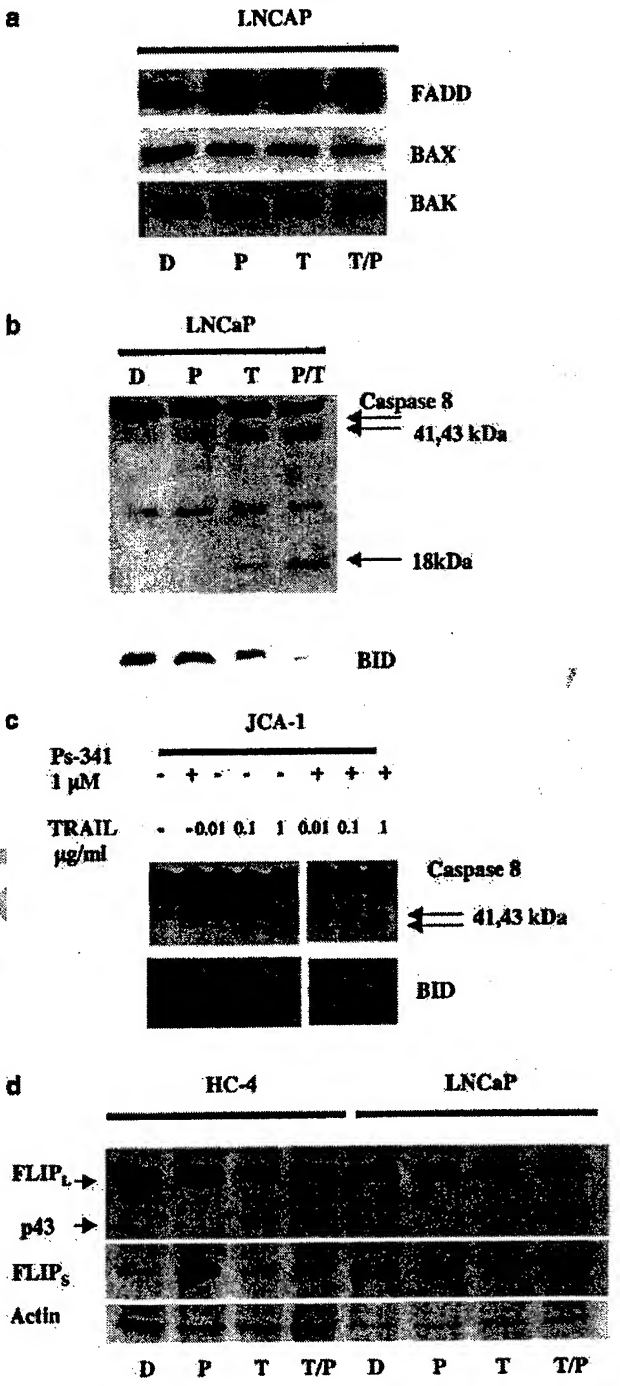

Figure 3 TRAIL and PS-341 treatment increase the levels of activated caspase 8 and cleaved Bid. (**a**) Levels of proapoptotic proteins in LNCaP cells. LNCaP cells were treated with vehicle (DMSO), PS-341 (1 μM), TRAIL (100 ng/ml), or both agents for 18 h, and extracts of these cells Western blotted with antibodies to BAX, BAK, and FADD. (**b**) Activation of caspase 8 by TRAIL and PS-341. LNCaP human prostate cancer cells were treated overnight with DMSO (D), PS-341 (1 μM) (P), TRAIL (100 ng/ml) (T), or a combination of both agents (T/P). (**c**) JCA-1 human bladder cancer cells were treated with varying concentrations of TRAIL and/or PS-341 (1 μM). Extracts were Western blotted with antibodies to either Bid or caspase 8. The arrows identify known caspase 8 cleavage products. (**d**) Response of cFLIP to PS-341 and TRAIL. HC-4 human colon cancer cells and LNCaP human prostate cancer cells were treated for 4 h (HC-4) or 6 h (LNCaP) with DMSO (D), PS-341(1 μM) (P), TRAIL(100 ng/ml), (T), or the combination (T/P). Cell extracts were Western blotted with NF6 antibody to cFLIP

combination of PS-341 and TRAIL. These data do not suggest a role for c-FLIP in PS-341-mediated sensitization to TRAIL.

It was shown that after treatment with TRAIL, HCT-116, but not HC-4 (Bax negative), cells responded with mitochondrial release of SMAC protein (Deng *et al.*, 2002). However, HC-4 cells transfected with SMAC were able to undergo TRAIL-induced cell death, demonstrating that SMAC is sufficient for TRAIL-induced death in these cells (Deng *et al.*, 2002). The addition of TRAIL alone to HC-4 and HCT-116 causes similar levels of caspase 8 and Bid cleavage (Deng *et al.*, 2002, Figure 4a), suggesting that the difference in the ability of these cells to die may be regulated by levels of Bax. In contrast, others have shown a difference in the extent of cleavage of caspase 8 by TRAIL in these two cell lines (LeBlanc *et al.*, 2002). As in LNCaP and JCA-1 cells, the combination of PS-341 and TRAIL induced increased cleavage of caspase 8 and Bid (Figure 4a). As expected, neither TRAIL nor PS-341 addition in isolation was sufficient to cause release of SMAC (Figure 4a, 16) or cytochrome *c* (data not shown; Deng *et al.*, 2002) from HC-4 cells. However, the combination caused rapid release of SMAC, detectable in 1 h and with continued release up to 4 h (Figure 4b) after treatment.

The requirement for SMAC protein in TRAIL-induced death of HC-4 cells prompted us to ask whether PS-341 required cytochrome *c*-activated caspase 9 to potentiate TRAIL-induced death. For this purpose, we used caspase 9-deficient MEF (Hakem *et al.*, 1998). Wild-type MEF release cytochrome *c* and SMAC in response to combination treatment with PS-341 and TRAIL (data not shown). To test whether the absence of caspase 9 would block the effect of combined agents, wild-type and caspase 9-deficient MEF were exposed for 18 h to TRAIL, PS-341, or the combination, and analysed for cell death by DAPI uptake. As shown in Figure 5, both wild-type and caspase 9-deficient MEF underwent apoptosis to a similar degree in response to combination treatment of PS-341 and TRAIL, indicating that caspase 9 is dispensable for the potentiating effect.

To evaluate whether increased activation of caspase 8 by TRAIL and PS-341 was sufficient to induce cell death, we transfected HC-4 and HCT-116 cells with a vector that expressed the catalytic portion of the caspase 8 molecule cloned in frame with three repeats of the FK506 binding protein (FKBP12) (Fkp-caspase 8) (Yang *et al.*, 1998). Overexpression of this protein in the absence of crosslinking was sufficient to induce apoptotic cell death in 293 cells (data not shown), although crosslinking of the FK506 sequences enhances the ability of this protein to induce cell death (Yang *et al.*, 1998). We used X-Gal staining of LacZ-transfected cells as a marker of those cells expressing transfected Fkp-caspase 8. We found that the overexpression of activated caspase 8 in HC-4 cells was able to induce apoptosis and that the level of apoptosis, although slightly less than in HCT-116, was not

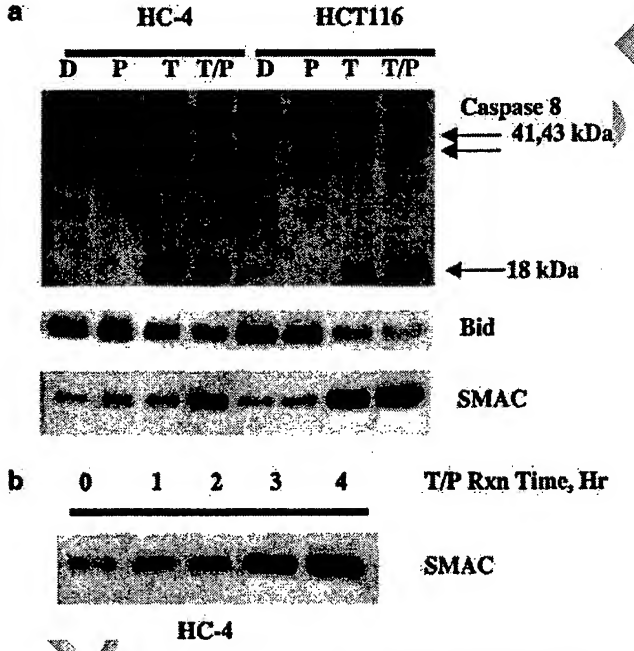

Figure 4 PS-341 enhances the ability of TRAIL to release SMAC from Bax-deficient HC-4 cells. (**a**) SMAC is released from HC-4 cells by the combination of TRAIL and PS-341. HC-4 and HCT-116 were treated for 3 h with DMSO (D), TRAIL (T) (100 ng/ml), PS-341 (P) (1 μM) or both (T/P) agents. Extracts of these cells were run on 12% SDS : PAGE gels and Western blotted with antibodies to the indicated proteins. (**b**) HC-4 cells rapidly release SMAC after the addition of TRAIL (100 ng/ml and PS-341 (1 μM) (T/P). HC-4 cells were treated with both compounds for varying periods of time as shown. Extracts of these cells were treated as in (**a**)

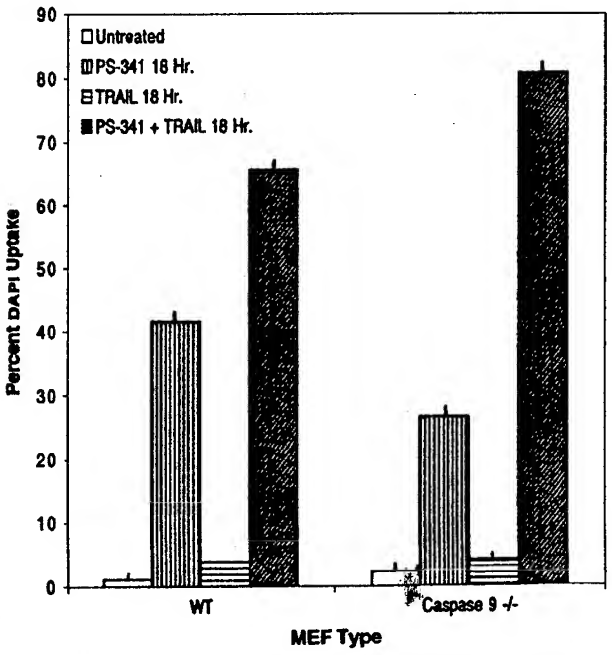

Figure 5 Response of caspase $9^{-/-}$ MEF to PS-341 and TRAIL. Caspase $9^{-/-}$ MEF were exposed to vehicle, 0.1 μM PS-341, 1 μg/ml TRAIL, or the combination for 18 h. Cells were processed for DAPI uptake as described in Materials and methods. Data are expressed as mean and range of two independent experiments

significantly different than in this parental cell line (Figure 6). Thus, in the absence of Bax, increased levels of activated caspase 8 are sufficient to induce apoptosis of these colon cancer cells.

Since the combination of PS-341 and TRAIL increased caspase 8 activation, we evaluated the ability of PS-341 to affect the levels of TRAIL receptors DR5 and DR4. HC-4 cells were incubated with PS-341 alone for varying periods of time. The cells were then either incubated with histidine-tagged TRAIL and lysed to measure cell surface receptors, or lysed followed by addition of histidine-tagged TRAIL to measure whole-cell receptor levels. Both DR5 and DR4 receptors were then immunoprecipitated with antihistidine antibody. Controls were not treated with histidine-tagged TRAIL, although the rest of the procedure was followed. In contrast to PS-341's lack of effect on levels of TRAIL pathway proteins downstream from TRAIL receptors, incubation of HC-4 cells with PS-341 alone resulted in marked accumulation of TRAIL receptor DR5 and to a lesser extent DR4 both in whole-cell lysates and (as shown, Figure 7a) on the cell surface protein. After 3 h treatment with PS-341, approximately the time HC-4 cells with apoptotic morphology begin to appear in cultures treated with the combination of PS-341 and TRAIL, DR5 receptors are reproducibly elevated ($51 \pm 13\%$, $n=4$), while 18 h treatment with PS-341 raised DR5 levels 4–8-fold. In comparison, DR4 levels did not increase appreciably by 3 h although an approximate doubling was observed after 18 h. Similar changes in DR5 and DR4 were obtained with treatment of PC-3 human prostate cancer cells with PS-341 (data not shown).

To determine whether the observed increases in these proteins were due to transcriptional activation, semi-quantitative and quantitative real-time PCR was performed as described in Materials and methods. This technique was chosen over Northern analysis to avoid complications arising from the high degree of sequence similarity between the receptors (MacFarlane *et al.*, 1997). Figure 7b shows semiquantitative RT–PCR of cDNA from HC-4 cells exposed to PS-341 for varying amounts of time, using primers specific for DR5. The same cDNAs were subjected to quantitative real-time PCR and the ΔCt values (relative to cDNA from control cells) are indicated beneath the corresponding lanes. The results indicate an early increase in DR5 mRNA transcripts of about twofold, consistent with the observed rise in DR5 protein, with maximal increases of 6–8-fold at 6 and 12 h followed by a return to baseline.

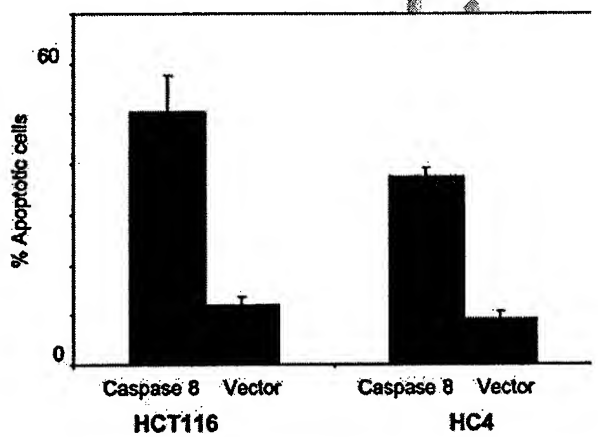

Figure 6 Overexpression of caspase 8 induces apoptosis in HC-4 and HCT-116 cells. HC-4 and HCT-116 were transfected with a plasmid containing encoding the LacZ cDNA with or without equivalent amounts of Fkp3-caspase 8 (180) or carrier DNA. After 24 h, later the plates were stained with X-Gal. In all, 10 fields in each well were scored for total blue and apoptotic blue cells (approx. 200 total blue cells/well). The s.d. of the mean of two transfection experiments performed in triplicate is shown

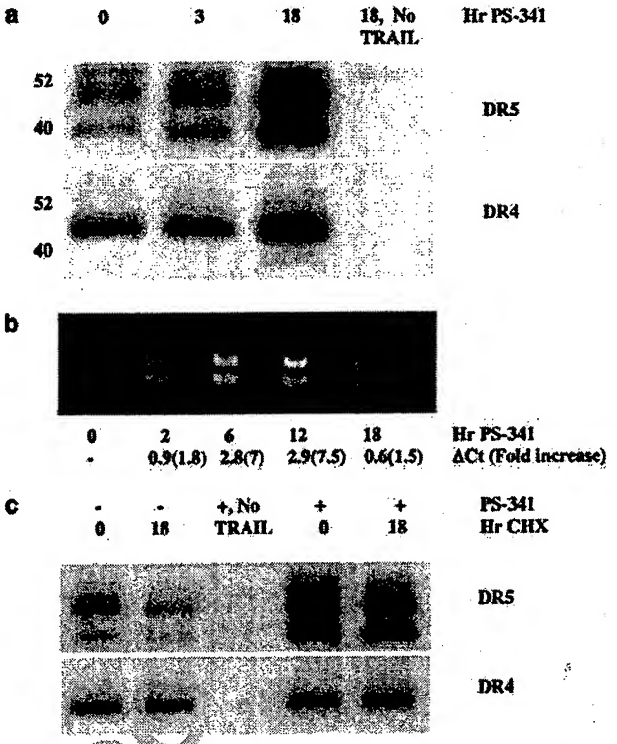

Figure 7 Response of TRAIL receptor protein and mRNA expression to PS-341 treatment. **(a)** PS-341 treatment increases cell surface DR5 and DR4 receptors. HC-4 cells were exposed to 1 μM PS-341 for 0, 3, and 18 h. Cell surface TRAIL receptors were prepared as described in Materials and methods and analysed by Western blotting for DR4 and DR5 protein. The lane labeled 'No TRAIL' represents cells to which histidine-tagged TRAIL was not added prior to cell lysis and incubation with antihistidine agarose (cf. Materials and methods). **(b)** DR5 transcript levels increase in response to PS-341 treatment. Semiquantitative PCR (gel) and quantitative real-time PCR (Δ Ct) were performed on cDNA prepared from RNA of HC-4 colon cancer cells exposed to PS-341 (1 μM) for various times. ΔCt values (the difference in cycle number at a given threshold during the linear phase of amplification) are relative to control cells; fold increases ($2^{\Delta Ct}$) are in parentheses. **(c)** PS-341 treatment prevents degradation of DR4 and DR5 protein. HC-4 cells were exposed to vehicle or PS-341 for 6 h. At this point, cycloheximide (50 μM) was added and incubation was continued for an additional 18 h. Total cellular TRAIL receptors were isolated as described in Materials and methods and subjected to Western analysis for DR4 and DR5. The lane labeled 'No TRAIL' is as in **(a)**

To address the possibility that PS-341 increased protein stability and thus contributed to accumulation of DR4 and DR5, receptor levels were determined following exposure of HC-4 cells to cycloheximide. An example of such an experiment is shown in Figure 7c. In the presence of PS-341, DR5 levels were unchanged after 18 h of cycloheximide treatment; in the absence of PS-341, levels decreased by 2.5-fold, suggesting that PS-341 treatment prevented the degradation of this protein. Attempts to determine the half-life of DR4 and DR5 by pulse-chase experiments utilizing ^{35}S-methionine proved difficult to interpret due to nonspecific binding of labeled proteins to the antihistidine antibody in the critical molecular weight range. Thus, our data suggest transcriptional activation at early time points, and protein accumulation secondary to decreased degradation.

The increased stability of DR5 in the presence of PS-341 prompted us to examine whether this receptor might be ubiquitinated. To investigate this possibility, a ELAG-tagged DR5 construct containing an inactivating mutation in the death domain to prevent the induction of apoptosis was cotransfected into 293 T cells with a cDNA expressing HA-tagged ubiquitin. Following treatments, DR5 was recovered by immunoprecipitation with anti-FLAG antibody coupled to agarose beads and analysed by Western blotting. The results are shown in Figure 8. High molecular weight forms of DR5 were detected with antibodies against HA, and were greatly increased by treatment with PS-341, thus demonstrating the enhanced ubiquitination of this protein.

Since HC-4 cells are transformed human colon cancer cells and might contain additional mutations in the apoptotic pathway in addition to the lack of Bax, we wanted to assess the sensitivity of normal cells, also devoid of Bax, to this combination of agents. In addition, it was reported that either Bax or Bak can mediate sensitivity to tBid-induced apoptosis (Wei *et al.*, 2001), suggesting that cells devoid of Bak should have a similar phenotype to those that were Bax-negative. We evaluated the response of MEF deficient in one or both of these proteins to PS-341, TRAIL, or the combination. Induction of apoptosis was evaluated by estimating the amount of PARP cleavage after 6 h treatment

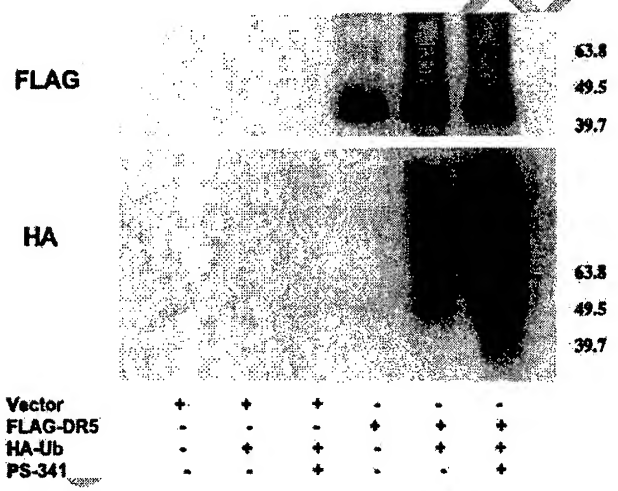

Figure 8 Ubiquitination of DR5. Expression vectors containing FLAG-tagged DR5 (L334N) (FLAG-DR5), HA-tagged ubiquitin (HA-Ub), or empty vector were transfected into 293 T cells in the combinations indicated. After 24 h, PS-341 (1 μM) or vehicle was added and the cultures were incubated overnight. Cell lysates were incubated overnight with anti-FLAG antibody covalently coupled to agarose beads at 4°. Bound material was eluted and prepared for Western blotting as described for DR5 immunoprecipitation. Blots were stained for DR5 (FLAG) or ubiquitin (HA) as indicated

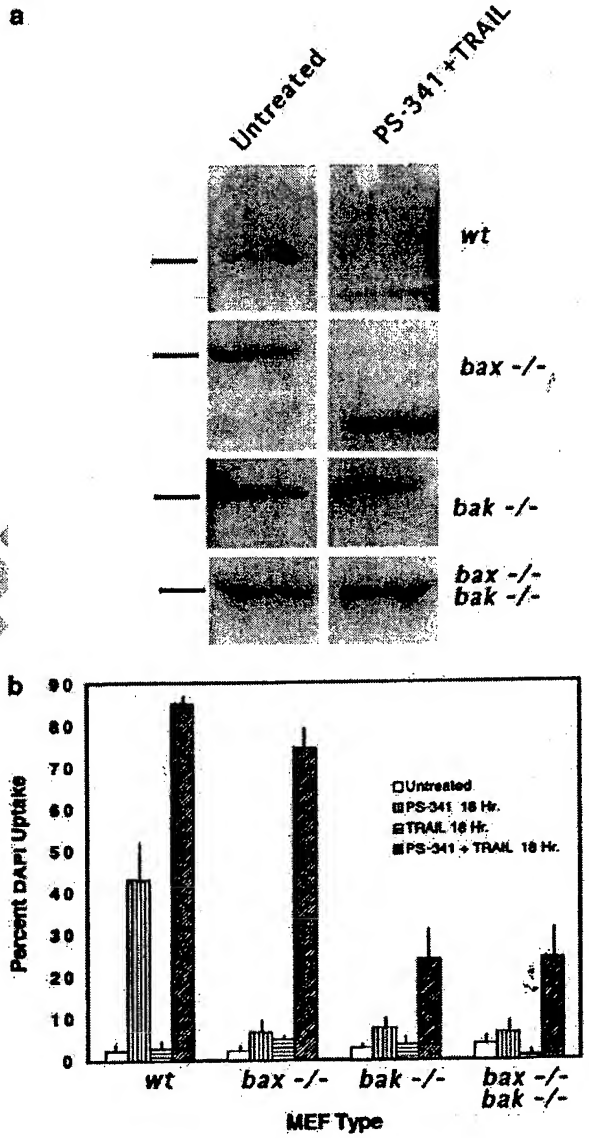

Figure 9 MEF response to PS-341 and TRAIL. (**a**) Combined treatment causes PARP cleavage in *wt* and *bax*$^{-/-}$ but not *bak*$^{-/-}$ MEF. MEF of the indicated genotype were exposed to vehicle, or the combination treatment of 0.1 μM PS-341, 1 μg/ml TRAIL for 6 h. Cells were lysed and extracts analysed for PARP expression and cleavage by Western blotting. The dark line at left indicates the position of uncleaved PARP (116 kd). (**b**) *Bak*$^{-/-}$ cells are less sensitive to combined treatment with PS-341 and TRAIL. MEF of the indicated genotype were exposed to vehicle, 0.1 μM PS-341, TRAIL, or the combination for 18 h. Cells were processed for DAPI uptake as described in Materials and methods. Data are expressed as mean and range of two independent experiments

(Figure 9a) or by uptake of the fluorescent dye DAPI after 18 h treatment (Figure 9b). A total of 6 h was chosen to evaluate PARP cleavage, because by 18 h a large number of cells were apoptotic. After 6 h, PARP cleavage is essentially complete in wild-type (*wt*) and $bax^{-/-}$ MEF treated with the combination of PS-341 and TRAIL. At this time, less than 5% of *wt* and $bax^{-/-}$ cells took up DAPI irrespective of treatment. After 18 h treatment with the combination of PS-341 and TRAIL, 75% or more *wt* and $bax^{-/-}$ cells took up DAPI and demonstrated an apoptotic nuclear morphology. In contrast to the data obtained with $bax^{-/-}$ fibroblasts, at 6 h in the $bak^{-/-}$ or $bax^{-/-}$ $bak^{-/-}$ cells, there was no evidence of PARP cleavage in cells treated with the combination of PS-341 and TRAIL; and only 25% of $bak^{-/-}$ or $bax^{-/-}$ $bak^{-/-}$ cells underwent apoptosis after 18 h as evidenced by DAPI uptake. Thus, cells lacking Bak appear to be significantly more resistant to combination treatment with PS-341 and TRAIL.

Discussion

The ability of PS-341 to enhance TRAIL-induced cell death is not tumor-type specific and was seen by us in LNCaP cells; two additional prostate cancer cell lines, DU-145 and PC-3; in the bladder cancer cell lines JCA-1 and TSU-PR (Van Bokhoven *et al*., 2001); in the colon cancer cell line HC-4; and in MEF. The ability of proteasome inhibitors, including PS-341, to stimulate TRAIL-induced apoptosis has also been reported in other cell lines (Mitsiades *et al*., 2001; Franco *et al*., 2001).

The effects of TRAIL on induction of apoptosis in human prostate cancer cells are known to be blocked by overexpression of Bcl-2 or Bcl-xL (Munshi *et al*., 2001; Roklin *et al*., 2001). However, we found that the combination of PS-341 and TRAIL overcame the block to mitochondrial participation in the apoptotic process mediated by overexpression of Bcl-xL. We obtained similar results in cells deficient in Bax or caspase 9. The mechanism allowing this effect appears to originate in part with the ability of PS-341 to increase the levels of both DR5 and DR4 receptors, leading to enhanced cleavage of caspase 8 and Bid, and increased release of SMAC and cytochrome *c*. Since this combination of agents is active in caspase 9-deficient MEF, it is likely that the apoptosome including cytochrome *c*, APAF-1, and caspase 9 is not needed for this effect and activated caspase 8 cleaves caspases 3 and 7 directly leading to the induction of apoptosis. Other agents, including sulindac (He *et al*., 2002), and chemotherapeutic agents etoposide and camptothecin (Gibson *et al*., 2000; Nagane *et al*., 2000; Lacour *et al*., 2001; LeBlanc *et al*., 2002), have been shown to enhance the ability of TRAIL to kill Bax-negative HC-4 cells (LeBlanc *et al*., 2002) presumably by inducing increases in DR5 but not DR4 TRAIL receptors. Quantitative PCR indicates that PS-341 induces a twofold increase in mRNA for DR5 at 2 h after treatment of HC-4 cells, increasing to eightfold by 12 h, suggesting that PS-341 controls the levels of DR5 in part through transcriptional mechanisms. Array experiments carried out by others after chemotherapy treatment of HC-4 cells have shown a similar level of increase in DR5 (LeBlanc *et al*., 2002), but not DR4, while PS-341 treatment of myeloma cells caused no change in mRNA levels at 2 and 4 h but did induce an increase at 8 h (Mitsiades *et al*., 2002).

The ubiquitin–proteasome pathway acts as a regulator of endocytosis of selected membrane receptors, for example, the growth hormone and epidermal growth factor receptors, and controls lysosomal degradation (Govers *et al*., 1999; Longva *et al*., 2002) of these receptors. It was shown that degradation of many ubiquitinated membrane proteins was slowed in the presence of proteasome inhibitors (van Kerkhov and Strous, 2001) and that mutational inactivation of the proteasome pathway prevented endocytosis of the growth hormone receptor and increased its abundance on the cell surface (van Kerkhov *et al*., 2002). DR5 is known to be internalized into endosomes following exposure of cells to TRAIL (Zhang *et al*., 2000). Our findings that DR5 is ubiquitinated and that PS-341 increased its stability suggest that similar mechanisms may be involved for these death receptors. Thus, PS-341 may enhance the number of TRAIL receptors both through protein stabilization and transcriptional mechanisms.

The effects of PS-341 on c-FLIP is of interest due to accumulating evidence of the importance of c-FLIP in receptor-mediated apoptosis. Both c-$FLIP_L$ and c-$FLIP_S$ have been shown to inhibit receptor-mediated apoptosis, apparently by different mechanisms (reviewed in Krueger *et al*., 2001). Our data indicate that PS-341 does not downregulate levels of either form of FLIP, and that the combination of PS-341 and TRAIL does not alter the degree of c-$FLIP_L$ cleavage over that induced by TRAIL alone, even though caspase 8 cleavage is increased (Figures 3b,c and 4a), It could be that FLIP is limiting while caspase 8 is not, thus allowing for greater caspase 8 activation in response to PS-341-induced upregulation of TRAIL receptors. Other mechanisms are possible, for example, c-FLIP phosphorylation (Higuchi *et al*., 2003).

Experiments examining the ability of PS-341, TRAIL, or the combination of these agents to induce apoptosis in MEF missing Bax, Bak, or both proteins suggests an important role for these proteins in regulating apoptosis induced by these agents. Interestingly, wild-type MEF underwent moderate levels of apoptosis when incubated with PS-341 alone, but MEF missing either Bax or Bak were resistant. The mechanism by which PS-341 induces apoptosis is unknown. Suggestions have been made that modulation of NF-κB plays a role in its action (Wang *et al*., 1998). Recent array data (Mitsiades *et al*., 2002) suggest that PS-341 upregulates the levels of a number of caspases and decreases the levels of Bcl-2. Clearly, from these data the mitochondrial death pathway is necessary for PS-341-induced apoptosis. It is possible that PS-341 treatment activates a BH3 protein that binds to Bak or Bax to induce mitochondrial release of cytochrome *c* and other proapoptotic proteins.

We found that treatment with the combination of TRAIL and PS-341, as opposed to treatment with these compounds individually, triggered apoptosis much more effectively in MEF containing Bak. The combined treatment was significantly less effective in MEF lacking Bak, or both Bak and Bax. One explanation for our results is that a PS-341-induced increase in Bak protein is needed to enhance TRAIL-mediated apoptosis. The addition of etoposide to HC-4 cells was shown to stimulate a marked increase in Bak mRNA, although the actual protein levels were not measured (LeBlanc *et al.*, 2002). However, we observed no increase in Bak protein with PS-341 treatment in multiple cell types including LNCaP, HC-4, and MEF (data not shown). Whether the functions of Bak and Bax are completely interchangeable is not known. Bak-deficient T cells were resistant to apoptosis induced by staurosporine, etoposide, and bleomycin (Wang *et al.*, 2001) and failed to release cytochrome *c* when transfected with Bid. Mandic *et al.* (2001) found that cisplatin induced the proapoptotic conformation of Bak, but not of Bax, in three out of four melanoma cell lines, suggesting some specificity in signaling to Bak. However, in baby mouse kidney cells, the presence of either Bak or Bax was sufficient to allow for TNF α-induced apoptosis (Degenhardt *et al.*, 2002). Both Bax or Bak-deficient MEF were sensitive to the killing effects of tBid (Wei *et al.*, 2001), the activated form of Bid, and BimS (Zong *et al.*, 2001), suggesting that these proteins were interchangeable in inducing the death pathway. It is possible that PS-341 regulates a BH3 domain protein which preferentially binds to Bak to induce apoptosis. Further experiments are needed to clarify this hypothesis.

Our data suggest a model whereby PS-341 enhances TRAIL killing by increasing the level of DR5 and DR4 receptors, thus increasing caspase 8 activation. As suggested by the induction of apoptosis in *bax*$^{-/-}$ *bak*$^{-/-}$ and caspase 9-deficient MEF, mitochondria may not be necessary for apoptosis induced by PS-341 and TRAIL; caspase 8 may directly cleave caspases 3 and 7 causing cell death. In the presence of Bak, however, the mitochondria are activated to release cytochrome *c*, SMAC, and other proteins that serve to amplify the signal from TRAIL receptors. The observation that caspase 9-deficient MEF are still responsive to this combination is consistent with previous experiments (Deng *et al.*, 2002), suggesting that SMAC upregulation alone is sufficient for increased sensitivity to TRAIL. SMAC would function to further increase the activation of caspases 3 and 7.

Our data demonstrate that the combination of PS-341 and TRAIL should be active in treating human tumors with multiple abnormalities in the apoptotic cascade.

Abbreviations

TRAIL, TNF-like apoptosis-inducing ligand; DISC, death-inducing signaling complex; PARP, polyADP ribose polymerase; MEF, murine embryonic fibroblasts.

Acknowledgements

We thank Dr Sookhee Bang (National Institutes of Health, Bethesda, MD, USA) for preparing TRAIL protein. The HC-4 and HCT-116 cells were kindly provided by both Drs B Vogelstein (Johns Hopkins University, Baltimore MD, USA) and M Perucho (Burnham Institute, San Diego, CA, USA). We also thank Dr David Baltimore (California Institute of Technology, Pasadena, CA, USA) for providing the Fkp3-caspase 8 (180) cDNA construct. The LNCaP-Bcl-xL cells were a gift of Dr Ralph Buttyan (Columbia Presbyterian Medical Center, New York, NY, USA). We are grateful to Dr R Hakem (Ontario Cancer Institute, Toronto, Canada) for providing the caspase 9-deficient fibroblasts. We thank Dr Daniel Chan for performing the Calculyn analysis, examining synergism between these two compounds. The NF6 antibody against c-FLIP was a kind gift of Dr Marcus Peter, Ben May Institute for Cancer Research, University of Chicago, Chicago, IL, USA. We thank Dr Mathias Treier, Max Delbrueck Centrum, Berlin, Germany for the HA-tagged ubiquitin expression construct. The continued support of this project by Millenium Corp. is highly appreciated. Wei-Xing Zong is a recipient of the Cancer Research Institute (CRI) postdoctoral fellowship.

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Classification: Mechanisms of Signal Transduction

Oncogenic Ras Sensitizes Human Cells to TRAIL-Induced Apoptosis by Facilitating Caspase 8 Recruitment

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Running Title: Oncogenic Ras Sensitizes Normal Human Cells to TRAIL

SUMMARY

TRAIL is a cytotoxic cytokine that induces apoptosis in tumor cells but rarely kills normal ones. To determine how normal human cells acquire TRAIL-sensitive phenotype during the process of malignant transformation we used an experimental system that allows for controlled conversion of human cells from normal to cancerous by introduction of several genes. Human embryonic kidney cells and foreskin fibroblasts were first immortalized by the combination of the early region of simian virus 40 and telomerase, and then transformed with oncogenic Ras. Both normal and immortalized cells were resistant to TRAIL-induced apoptosis, whereas Ras-transformed cells were susceptible. Ras transformation enhanced TRAIL-induced activation of caspase 8 by increasing its recruitment to TRAIL receptors. The pro-apoptotic effects of Ras could be reversed by inhibitors of either farnesyl transferase or MEK. The expression of constitutively activated MEK1 enhanced caspase 8 recruitment and sensitized immortalized human embryonic kidney cells to TRAIL. These results indicate that in normal human cells the TRAIL-induced apoptotic

signal is blocked at the level of caspase 8 recruitment and that this block can be eliminated by Ras transformation, involving activation of the MAP kinase pathway.

INTRODUCTION

TRAIL (tumor necrosis factor alpha-related apoptosis inducing ligand) is a cytotoxic cytokine that selectively induces apoptosis in a variety of tumor cells but rarely affects normal ones (1). At least five receptors for TRAIL have been identified. Two of these, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) contain a conserved motif, the death domain and signal apoptosis (2). Binding of TRAIL to DR4 or DR5 induces formation of the death-inducing signaling complex (DISC), which is comprised of the adaptor protein FADD/MORT1 and the FADD-binding cysteine protease, caspase 8/FLICE (3,4). The formation of the DISC triggers proteolytic autoprocessing and activation of caspase 8, which in turn cleaves and activates the downstream caspases, 3, 6 or 7, leading to apoptosis. In addition, caspase 8 cleaves a cytolinker, plectin, inducing the reorganization of the cytoskeleton (5), and the pro-apoptotic protein, Bid, generating a cleavage product that triggers the release of cytochrome C from mitochondria (6,7).

It remains unclear as to why TRAIL is selective toward cancer cells despite the ubiquitous expression of its receptors in normal tissues. Malignant transformation is believed to require stepwise accumulation of at least three genetic alterations: inactivation of tumor suppressors,

immortalization, and receipt of a continuous mitogenic signal (8). To investigate at which step of this process and how human cells acquire a TRAIL-sensitive phenotype we used a recently developed experimental system that mimics stepwise progression of human cells from normal to tumorigenic (9). Normal cells were converted to tumorigenic by serial introduction of the early region of simian virus 40 (SV40ER) that disables tumor suppressors p53 and Rb, catalytic subunit of telomerase (hTERT) to ensure their unlimited lifespan and oncogenic allele of Ha-Ras (H-*ras*-V12) that provides cells with continuous growth signal. Upon transformation with activated Ras these cells become capable of anchorage-independent growth and formation of tumors in immunodeficient mice (9). This model allowed us to investigate a mechanism by which malignant transformation renders human cell susceptible to TRAIL-induced apoptosis.

EXPERIMENTAL PROCEDURES

Reagents.

The following reagents were obtained from the indicated sources: SCH 66336 (Schering-Plough Research Institute; PD 98059 and U0126 (Calbiochem); z-VAD-FMK (Enzyme System Products); anti-phospho-ERK and anti-ERK (New England

Biolabs); anti-Bid (Zymed Laboratories); anti-plectin (Transduction Laboratories) anti-caspase 8 and anti-FADD (Upstate Biotechnology); monoclonal antibody to caspase 10 (Clone 4C1, MBL International); anti-DR4 and anti-DR5 (Alexis); monoclonal antibody NF6 to FLIP (a gift of Marcus Peter, Ben May Institute for Cancer Research). The expression and purification of TRAIL from yeast *P.pastoris* has been described in detail elsewhere (10).

Cell culture

Normal human foreskin fibroblasts (BJ) were obtained from the American Type Culture Collection. Normal human embryonic kidney cells (HEK) were kindly provided by Silvia Bacchetti (McMaster University, Hamilton, Ontario, Canada). Immortalization of the cells with SV40 large T antigen and hTERT and their subsequent transformation with H-*ras*V12 has been described in detail elsewhere (9). MEK^{Q56P} was expressed in immortalized HEK cells using pBabe-Puro vector (11) (a gift of Scott W. Lowe, Cold Spring Harbor Laboratory). Retroviral stocks were generated in Phoenix ecotropic packaging line (G. Nolan, Stanford University). Cells infected with an empty vector were used as a control. Human bladder cancer cell line T24 was kindly provided by Gary J. Miller (University of Colorado Health Sciences Center).

Cytotoxicity assays

Cell viability was determined using the tetrazolium-based Aqueous One assay (Promega). Detection of DNA fragmentation by agarose gel electrophoresis was performed using the Suicide-Track™ DNA Ladder Isolation Kit (Oncogene Research Products) using a procedure that selectively extracts apoptotic DNA from intact chromatin.

DISC immunoprecipitation

HEK cells grown in roller bottles (approximately 4 x 10^8 cells per condition) were scraped into 10 ml of conditioned medium, combined, precipitated and resuspended in 5 ml of the conditioned medium. As judged by the trypan blue exclusion assay, more than 80% of cells remained viable after this procedure. Stimulation with TRAIL was achieved by incubation with TRAIL (1 μg/ml) for 20 minutes at 37°C. The cells were washed in ice-cold PBS and lysed in 10 ml of Triton/glycerol/HEPES buffer (12), cleared by centrifugation and equalized for protein content. Precipitation of the unstimulated TRAIL receptors was achieved by adding TRAIL (1 μg/ml) to the lysates for 30 minutes at 4°C. The TRAIL receptors that were complexed with TRAIL were immunoprecipitated by the addition of 25 μl of anti-

polyhistidine agarose (Sigma) for 2 h at 4°C and bound proteins eluted with 100 mM glycine-HCl, pH 2.3 (2 times, 40 µl, 10 minutes 4°C).

RESULTS

Conversion of normal human cells to tumorigenic ones sensitizes them to TRAIL.

Human embryonic kidney epithelial cells (HEK) and foreskin fibroblasts (BJ) were first immortalized by the combination of SV40ER and hTERT and then transformed with oncogenic Ras (9). Both normal and immortalized cells remained resistant to TRAIL whereas the introduction of activated Ras rendered them susceptible to TRAIL-induced apoptosis, as assessed by both morphological changes typical of apoptosis (Fig. 1A) and DNA fragmentation (Fig. 1B).

To test whether the observed sensitization is specific to TRAIL we investigated how Ras transformation affects apoptosis mediated by TNFα, agonistic antibody to CD95/FAS, and kinase inhibitor staurosporine, a compound that triggers cell death by inducing the release of cytochrome C from mitochondria (13). As demonstrated by a cell viability assay (Fig. 1C) oncogenic Ras did not render cells more susceptible to apoptosis mediated by either Fas or TNFR and

even partially rescued them from staurosporine-induced toxicity.

Ras transformation potentiates TRAIL-induced activation of the initiator caspase 8.

As the process of immortalization did not sensitize cells to TRAIL-induced apoptosis, these cells were used as the control for further experiments. To determine what step in the TRAIL-mediated apoptotic cascade is enhanced by Ras transformation we compared TRAIL-induced proteolytic events in Ras-transformed and control, immortalized cells. As shown in Figure 2, the addition of TRAIL to control cells did not induce cleavage of the initiator caspase 8. In contrast, treatment of Ras-transformed cells with TRAIL induced significant caspase 8 cleavage. Interestingly, TRAIL-induced processing of another proximal caspase, caspase 10 (14) was not facilitated by Ras transformation (Figure 2), suggesting that these two enzymes may be regulated in different ways.

Caspase 8 cleavage is not necessarily accompanied by an increase in the activity of this proteolytic enzyme toward its cellular substrates (15). Therefore, we examined the ability of Ras to enhance TRAIL-induced cleavage of specific caspase 8 substrates, Bid (6,7) and plectin (5). As

shown in Figure 2, oncogenic Ras enhanced the ability of TRAIL to induce the cleavage of plectin in both HEK and BJ cells. Although we could not detect significant cleavage of Bid in BJ cells, proteolytic processing of this protein was evident in Ras-transformed HEK cells that were generally more responsive to TRAIL than BJ fibroblasts.

Ras transformation enhances recruitment of caspase 8 to TRAIL DISC.

Next, we examined how transformation of cells with Ras affected the ability of TRAIL receptors to form a functional DISC. To immunoprecipitate the activated TRAIL receptors, DR4 and DR5, we first incubated intact cells with a $(His)_6$-tagged recombinant TRAIL (10) and then immunoprecipitated the receptors with an anti-polyhistidine antibody. To immunoprecipitate unstimulated receptors, cells were first lysed with detergent and then $(His)_6$-TRAIL was added to the extracts.

These and subsequent experiments were performed using the HEK cells because the pro-apoptotic effects of Ras transformation were more robust in these cells. As shown in Figure 3, the antibody to $(His)_6$-TRAIL efficiently immunoprecipitated both TRAIL receptors, DR4 and DR5. The lack of immunoprecipitation of TRAIL receptors when the

cells or cell lysates were not treated with the $(His)_6$-TRAIL confirmed the specificity of this procedure. DR5 was detected as a doublet, corresponding to two known splice variants of this protein (16). This result demonstrates that oncogenic Ras does not significantly affect the level of DR4 in these cells but elevates the expression of DR5. Densitometry analysis of two independent experiments revealed the 1.5-2 fold increase of DR5 expression in Ras transformed versus control cells.

When cells were treated with TRAIL prior to lysis, two additional proteins co-immunoprecipitated with the TRAIL receptors, FADD and caspase 8 (Fig.3). FADD co-immunoprecipitated equivalently with TRAIL receptors from Ras-transformed and control cells. In contrast, caspase 8 was recruited to TRAIL receptors in Ras-transformed cells much more efficiently than in control cells. Densitometry analysis of two independent experiments demonstrated that Ras increased caspase 8 recruitment 8- fold and more. This result suggests that Ras transformation sensitizes cells to TRAIL by facilitating the binding of caspase 8 to FADD.

To rule out the possibility that the observed difference in the amounts of co-immunoprecipitated caspase 8 resulted from different rates of caspase 8 processing and subsequent dissociation from the DISC, cells were pre-

treated with the pan-caspase inhibitor, Z-VAD-FMK. Inhibition of caspase 8 activity, however, did not have a significant effect on the amounts of caspase 8 bound to the TRAIL receptors (Fig. 3).

The pro-apoptotic effects of Ras are reversible.

To test whether sensitization of cells to TRAIL by Ras transformation is a reversible process we used the farnesyl transferase inhibitor, SCH 66336, a compound that inhibits prenylation of Ras proteins and suppresses their biological activity (17). As shown in Figures 4A and 4B, pre-treatment of Ras-transformed HEK cells with SCH 66336 efficiently rescued them from TRAIL-induced death.

To test whether Ras-induced MAP kinase pathway contributes to the pro-apoptotic effect of this oncogene, we used two inhibitors of MEK, PD 98059 (18) and U0126 (19). Figures 4A and 4B demonstrate that the MEK inhibitors efficiently rescued Ras-transformed HEK cells from TRAIL. Both the MEK and farnesyl transferase inhibitors suppressed TRAIL-induced cleavage of caspase 8 (Fig. 4C). These compounds thus appear to specifically reverse the pro-apoptotic effect of Ras.

We next tested whether Ras-dependent sensitization of cells to TRAIL-induced apoptosis can also occur in

transformed cells obtained from cancer patients. For this purpose we used bladder cancer cell line T24 that expresses the oncogenic allele of Ha-Ras (H-ras-V12) and possess constitutively activated MAP kinase (20). Using farnesyl transferase inhibitor SCH 66336 or MEK inhibitor PD 98059 we found that inhibition of either Ras processing or MAP kinase activity suppressed TRAIL-induced caspase 8 cleavage in T24 cells (Figure 4D) and rescued them from TRAIL-mediated apoptosis (data not shown). This result confirms that sensitization of cells to TRAIL by oncogenic Ras can indeed take place in spontaneous human cancers.

The pro-apoptotic effects of Ras are mediated by MAP kinase pathway.

The results presented in Figure 4 suggested that the pro-apoptotic effects of Ras involve MAP kinase pathway. We therefore investigated whether activation of this pathway alone is sufficient to sensitize HEK cells to TRAIL. For this purpose we used a gain-of-function mutant of MEK1 ($MEK1^{Q56P}$), a dual-specificity protein kinase that phosphorylates and activates MAP kinase (11,21). Using an antibody that specifically recognizes the phosphorylated, activated form of MAP kinase we found that expression of

$MEK1^{Q56P}$ induced phosphorylation of MAP kinase to approximately the same level as that observed in Ras-transformed cells (Fig. 5A).

Figure 5B demonstrates that activation of MAP kinase pathway potentiates the same step in the TRAIL-induced apoptotic cascade as oncogenic Ras. Treatment of either Ras-transformed or $MEK1^{Q56P}$-expressing but not control cells with TRAIL induced cleavage of both caspase 8 and two caspase 8 substrates, $cFLIP_L$ and Bid.

The results presented in Figure 3 suggested that oncogenic Ras sensitizes cells to TRAIL by enhancing the recruitment of caspase 8 to TRAIL DISC. To confirm that this effect is mediated by the MAP kinase pathway, we analyzed DISC formation under conditions in which this pathway was either inhibited by PD 98059, the MEK inhibitor, or activated by the expression of $MEK1^{Q56P}$. TRAIL-TRAIL receptor complexes were immunoprecipitated from TRAIL-treated cells using an antibody to polyhistidine. As shown in Figure 5C, comparable amounts of FADD co-immunoprecipitated with TRAIL receptors from control, Ras-transformed, or $MEK1^{Q56P}$-expressing cells. In contrast, significantly more caspase 8 was co-immunoprecipitated with TRAIL receptors from Ras-transformed cells than from control cells. Pre-treatment of Ras-transformed cells with

the farnesyl transferase inhibitor, SCH 66336, or MEK inhibitor, PD 98058, reduced the amounts of co-immunoprecipitated caspase 8. The expression of constitutively active MEK increased the recruitment of caspase 8 to TRAIL receptors almost as efficiently as did oncogenic Ras. These results indicate that the observed enhancement of TRAIL receptor DISC formation in Ras-transformed cells is mediated by MAP kinase pathway.

DISCUSSION

Although selective killing of cancer cells by TRAIL has been reported in many studies (1), the molecular mechanisms behind this selectivity remain unclear. Research has been focused on a comparison of apoptotic signals induced by TRAIL in normal cells versus those obtained from cancer patients. These studies implicated several proteins, including the protease-deficient homologue of caspase 8, FLIP (22) and TRAIL decoy receptors, DcR1 and DcR2 (23), in resistance of normal cells to TRAIL. However, a firm correlation between the cellular expression of these proteins and TRAIL resistance has not been established (24,25).

Given the number of genetic alterations that occur during neoplastic transformation in humans (8) it is

difficult to single out individual changes that are responsible for the acquisition of the TRAIL-sensitive phenotype. Here we describe the use of genetically defined transformation to investigate how conversion of human cells from normal to tumorigenic renders them sensitive to TRAIL-induced apoptosis. This experimental model mimics the stepwise accumulation of genetic alterations that occurs in human cancers: inactivation of tumor suppressors, acquisition of an unlimited lifespan, and receipt of a continuous mitogenic signal (9). Both normal and immortalized cells were found to be resistant to TRAIL-mediated apoptosis, indicating that pre-malignant changes including inactivation of tumor suppressors and acquisition of an unlimited lifespan are not sufficient to sensitize cells to TRAIL. However, the subsequent conversion of immortalized cells to tumorigenic ones by activated Ras rendered them susceptible to TRAIL-mediated apoptosis.

Oncogenic Ras enhanced TRAIL-induced apoptosis by potentiating activation of the initiator caspase 8. This sensitization was accompanied by the increase in the level of one of pro-apoptotic TRAIL receptors, DR5. Upregulation of DR5 may, therefore, contribute to the enhanced sensitivity of Ras-transformed cells to TRAIL. It seems more likely, however, that the major mechanism by which

activated Ras sensitizes cells to TRAIL involves facilitating the recruitment of caspase 8 to TRAIL DISC. First, transformation of cells with Ras resulted in moderate, 1.5-2 fold upregulation of DR5, whereas recruitment of caspase 8 increased at least 8 fold. Second, the inhibition of either Ras processing or MAP kinase activity reversed both pro-apoptotic effects of Ras and caspase 8 recruitment without a concomitant decrease in the levels of DR5. Third, despite upregulation of DR5, the amount of FADD associated with TRAIL DISC was not increased in Ras-transformed cells. As the level of another TRAIL receptor, DR4 was not affected by Ras transformation, 1.5-2 fold upregulation of DR5 may only modestly increase the overall number of TRAIL receptors in Ras-transformed cells.

As oncogenic Ras appears to sensitize cells to TRAIL-induced apoptosis by facilitating DISC formation, we thought Ras transformation might have similar effect on apoptosis mediated by two related death receptors, TNFR and Fas. However, we did not observe any Ras-dependent sensitization of cells to apoptosis induced by either recombinant TNFα or agonistic antibody to Fas (Figure 1C). There can be several potential explanations for the difference in TNF, Fas and TRAIL signaling. For instance, it has been reported that oncogenic Ras suppressed Fas-

mediated apoptosis by inducing the methylation of Fas promoter and inhibiting the expression of this protein (26). Therefore, observed resistance of Ras-transformed cells to Fas-mediated apoptosis is likely to be secondary to the loss of Fas expression. In contrast, expression of TRAIL receptors appears to be regulated by Ras in a different way. As demonstrated by our data (Figure 3), activated Ras had no effect on the expression of one of TRAIL receptors, DR4, and even increased the levels of another TRAIL receptor, DR5. With regard to TNFα, this cytokine rarely induces apoptosis unless it is combined with protein synthesis inhibitors. Since TNFα is a strong activator of anti-apoptotic transcription factor NF-kB, NF-kB-mediated transcriptional activity is believed to protect cells from TNFα-induced apoptosis (27). Unlike TNFα, TRAIL does not consistently activate NF-kB and even in those cells where TRAIL-mediated activation of NF-kB is observed, this effect was far less profound than that mediated by TNFα (28). In our experiments cells were treated by pro-apoptotic cytokines in the absence of protein synthesis inhibitors, i.e., under conditions when TNFα rarely exhibits pro-apoptotic activity. Alternatively, it cannot be ruled out that resistance of Ras-transformed HEK and BJ

cells to apoptosis induced by Fas antibody or TNFα may be secondary to the lack of receptors for these cytokines.

As Ras is capable of inducing genomic instability (29) it might, in principle, sensitize cells to TRAIL by promoting irreversible genetic alterations that would disable certain anti-apoptotic pathways. Two observations make this scenario unlikely. First, the Ras-transformed cells used in our studies were found to be polyclonal (9). Second, the pro-apoptotic effects of Ras could be reversed by the inhibitors of either farnesyl transferase or MEK. Apparently, continuous Ras signaling is essential for Ras-transformed cells to maintain both the transformed phenotype (30) and sensitivity to TRAIL.

Our results indicate that the pro-apoptotic effect of Ras is mediated by the MAP kinase pathway. It has been reported recently that activation of this pathway suppressed TRAIL-induced apoptosis in HeLa cells (31). One possible explanation for this apparent discrepancy is that MAP kinase is capable of eliciting both pro-apoptotic (32,33) and pro-survival responses (34). Anti-apoptotic effect of MAP kinase appears to be cell type-specific. For instance, activation of MAP kinase by phorbol myristate acetate was shown to suppress receptor-mediated apoptosis in some, but not all, types of cells (35). A pro-survival effect of the

MAP kinase pathway has, at least in part, been attributed to MAP kinase-dependent upregulation of anti-apoptotic FLIP proteins (36). However, positive regulation of FLIP expression by MAP kinase pathway has been observed only in a limited set of human cells (37). Likewise, we did not observe any significant changes in the levels of FLIP proteins either upon activation of MAP kinase by Ras or constitutively activated MEK (Fig. 5B), or upon treatment of Ras-transformed BJ and HEK cells with MEK inhibitors (data not shown).

In summary, using human cells that were progressively converted from normal into tumorigenic we demonstrate that: i) pre-malignant changes including inactivation of tumor suppressors and immortalization are not sufficient to sensitize human cells to TRAIL; ii) transformation of the immortalized cells with growth-promoting oncogene H-ras-V12 renders them susceptible to TRAIL-induced apoptosis; iii) oncogenic Ras potentiates TRAIL-induced activation of the initiator caspase 8 by facilitating its recruitment to the TRAIL DISC; iv) pro-apoptotic effects of Ras are reversible and involve the MAP kinase pathway; and v) constitutive activation of MAP kinase sensitizes immortalized human cells to TRAIL. As aberrant activation of MAP kinases is often associated with a neoplastic phenotype (20) sustained

MAP kinase activity may potentially serve as an indicator of malignant transformation recognized by a TRAIL-based antitumor surveillance system.

ACKNOWLEDGMENTS

We would like to thank Drs. William C. Hahn and Robert A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA) for immortalized and Ras-transformed HEK and BJ cells, Dr. Scott W. Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) for MEK^{Q56P} and HA-Ras-V12 constructs, Dr. Silvia Bacchetti (McMaster University, Hamilton, Ontario, Canada) for normal human embryonic kidney (HEK) cells, Dr. W. Robert Bishop (Schering-Plough Research Institute, Kenilworth, NJ) for the farnesyl transferase inhibitor, SCH 66336, Dr. Marcus E. Peter (Ben May Institute for Cancer Research, Chicago, IL) for anti-FLIP antibody NF6, and Dr. Gary Nolan (Stanford University, CA) for the Phoenix packaging cell line. This work was supported by the Department of Defense grant, DAMD 17-01-1-0045 (to A.S.K.).

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FIGURE LEGENDS

Fig. 1. Transformation of HEK and BJ cells with Ras sensitizes them to TRAIL-induced apoptosis. The effects of TRAIL (1 μg/ml) on normal (N), immortalized (I) or Ras-transformed (R) HEK cells and BJ fibroblasts were assessed 72 hours after the addition of TRAIL. (A) The morphology of the plated cells was assessed, and representative photographs are shown. (B) The cells were harvested, and extracts processed by DNA laddering assay. DNA laddering was visualized by ethidium bromide staining of 2% agarose gels. Molecular weight markers are shown on the right. (C) Immortalized or Ras-transformed HEK cells and BJ fibroblasts were treated for 72 hours with TRAIL, agonistic antibody 2R2 to Fas or recombinant TNFα at concentration of 1 μg/ml. In parallel experiment cells were treated for 24 hours with 1μM of staurosporine (indicated as "St"). The

relative viability of cells was measured by the MTS tetrazolium conversion assay. The error bars represent standard deviations of mean for triplicate determinations.

Fig. 2. Transformation of cells with Ras enhances the ability of TRAIL to activate caspase 8. Control cells (immortalized cells infected with empty vector) or Ras-transformed HEK cells and BJ fibroblasts were treated for 72 hours with 1 μg/ml of TRAIL. Cell lysates were immunoblotted with antibodies specific for caspase 8, caspase 10, Bid, and plectin. Equal protein loading was confirmed by probing the blot with antibodies to FADD.

Fig. 3. Transformation of cells with Ras enhances the recruitment of caspase 8 to FADD. Control and Ras-transformed HEK cells were treated with $(His)_6$-tagged TRAIL (1 μg/ml) for 20 minutes at 37°C (indicated as "TRAIL before lysis"). The cells were lysed and TRAIL-TRAIL receptor complexes immunoprecipitated with monoclonal antibody to polyhistidine. To immunoprecipitate unstimulated receptors TRAIL was added to cells after lysis. Aliquots of the immunoprecipitated material were analyzed by immunoblotting using antibodies specific for DR5, DR4, FADD and caspase 8. DR5 is detected as a doublet, corresponding to the two

different splice variants of this protein (16). Equal amounts of protein in cell lysates was confirmed by probing with antibodies to FADD and caspase 8.

Fig. 4. The effects of small molecule inhibitors on TRAIL-induced apoptosis of Ras-transformed cells. (A, B) Ras-transformed HEK cells were pre-treated for 72 hours with farnesyl transferase inhibitor SCH 66336 (1μM), MEK inhibitors PD 98059 (100 μM) and U0126 (20 μM), or 0.1% DMSO as a control. Cells were then treated for an additional 72 hours with different concentrations of TRAIL. (A) The relative viability of cells was evaluated using the tetrazolium conversion assay. (B) The cells were harvested, and extracts processed by DNA laddering assay. DNA laddering was visualized by ethidium bromide staining of 2% agarose gels. (C) Ras-transformed HEK cells were pre-treated with one of the small molecule inhibitors as described above and then incubated with TRAIL (1 μg/ml) for 48 hours. Following this treatment, the cells were lysed, and immunoblotted with an antibody to caspase 8. A short exposure of the ECL-treated blot was used to visualize holocaspase 8 and a long exposure for its 18-kDa proteolytic fragment. (D) Human bladder cancer cells T24 were pre-teated for 72 hours with farnesyl transferase

inhibitor SCH 66336 (1μM), MEK inhibitor PD 98059 (100 μM) or 0.1% DMSO as a control. Where indicated, cells were then treated for an additional 24 hours with TRAIL (1 μg/ml). Following this treatment, the cells were lysed, and immunoblotted with an antibody to caspase 8.

Fig. 5 The pro-apoptotic effects of Ras are mediated by the MAP kinase pathway. HEK cells were infected by retrovirus expressing constitutively activated mutant of MEK, $MEK1^{Q56P}$. (A) Control cells (indicated as "C"), $MEK1^{Q56P}$-expressing cells (indicated as "M") or Ras-transformed cells (indicated as "R") were lysed and analyzed by immunoblotting with antibodies specific for phosphorylated MAP kinase or total MAP kinase. (B) Control, $MEK1^{Q56P}$–expressing or Ras-transformed cells were treated for 48 hours with of TRAIL (1 μg/ml). Cell lysates were immunoblotted with antibodies specific for caspase 8, FLIP, and Bid. Equal protein loading was confirmed by probing the blot with antibodies to FADD. (C). TRAIL receptors were immunoprecipitated with the antibody to polyhistidine from control (C), Ras-transformed (R) or $MEK1^{Q56P}$–expressing cells (M) as described in the legend to Figure 3A. Where indicated cultures were pre-treated for 72 hours with farnesyl transferase inhibitor SCH 66336 (1 μM) or MEK

inhibitor PD 98059 (100 μM). Aliquots of the immunoprecipitated material were analyzed by immunoblotting using antibodies specific for DR4, DR5, caspase 8 and FADD.

Figure 1

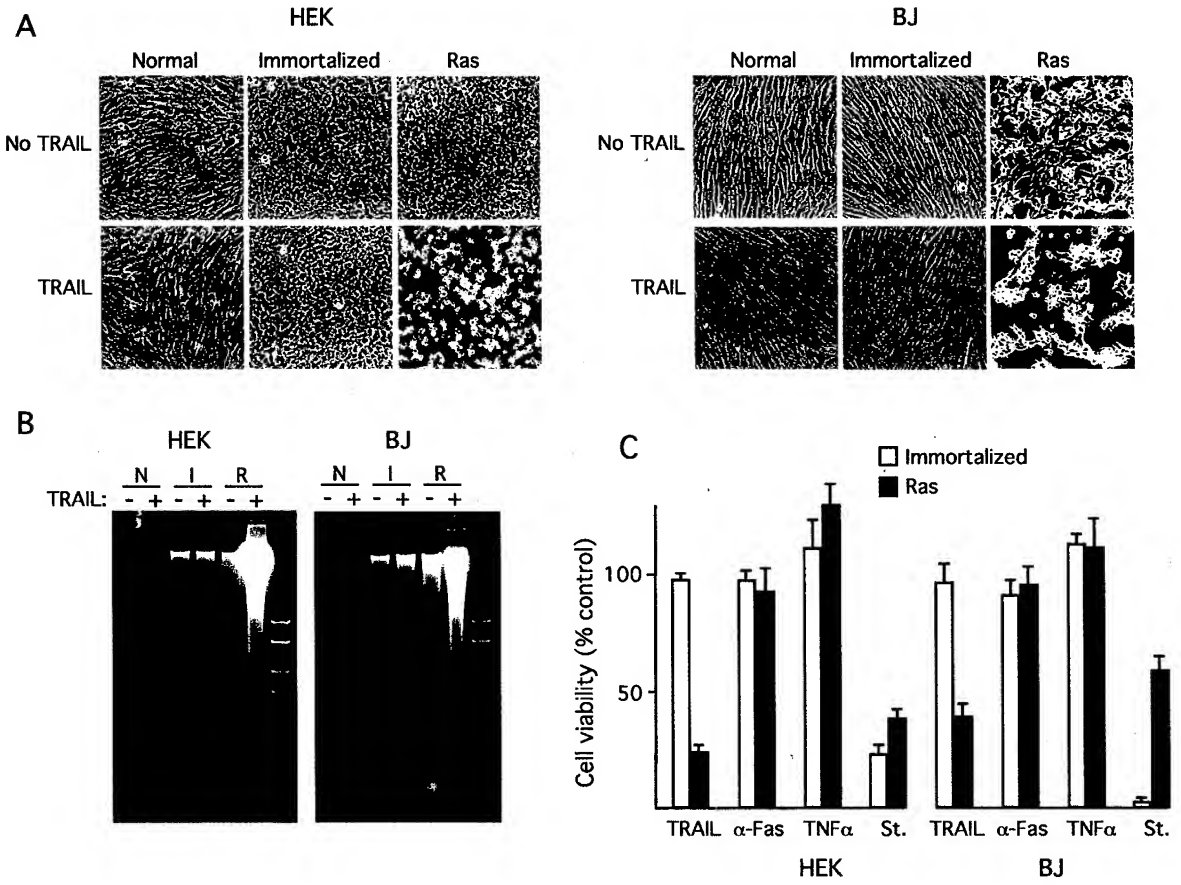

A
HEK
Normal
Immortalized
Ras
No TRAIL
TRAIL
BJ
Normal
Immortalized
Ras
No TRAIL
TRAIL
B
HEK
BJ
N I R
N I R
TRAIL: - + - + - +
- + - + - +
C
Immortalized
Ras
Cell viability (% control)
100
50
TRAIL α-Fas TNFα St.
TRAIL α-Fas TNFα St.
HEK
BJ

Figure 2

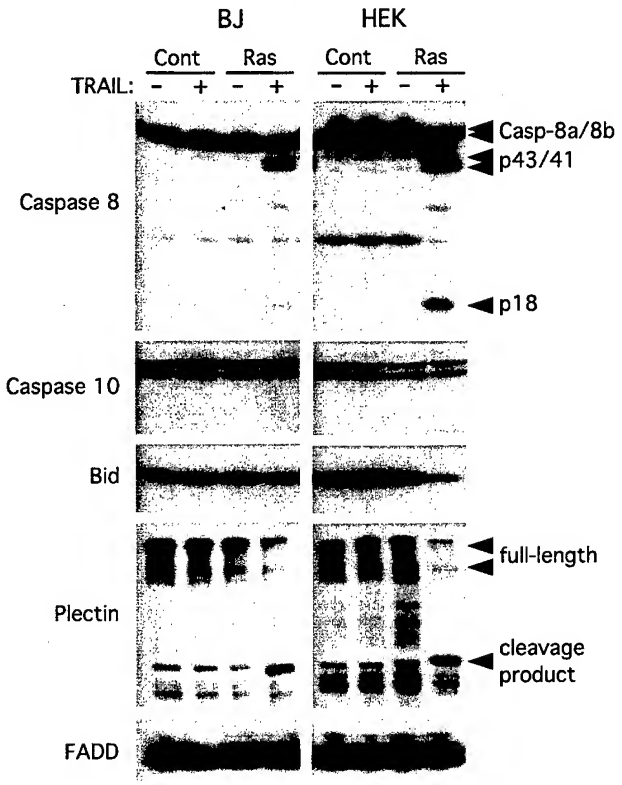

BJ
HEK
Cont
Ras
Cont
Ras
TRAIL: - + - + - + - +
Caspase 8
Casp-8a/8b
p43/41
p18
Caspase 10
Bid
Plectin
full-length
cleavage product
FADD

Figure 3

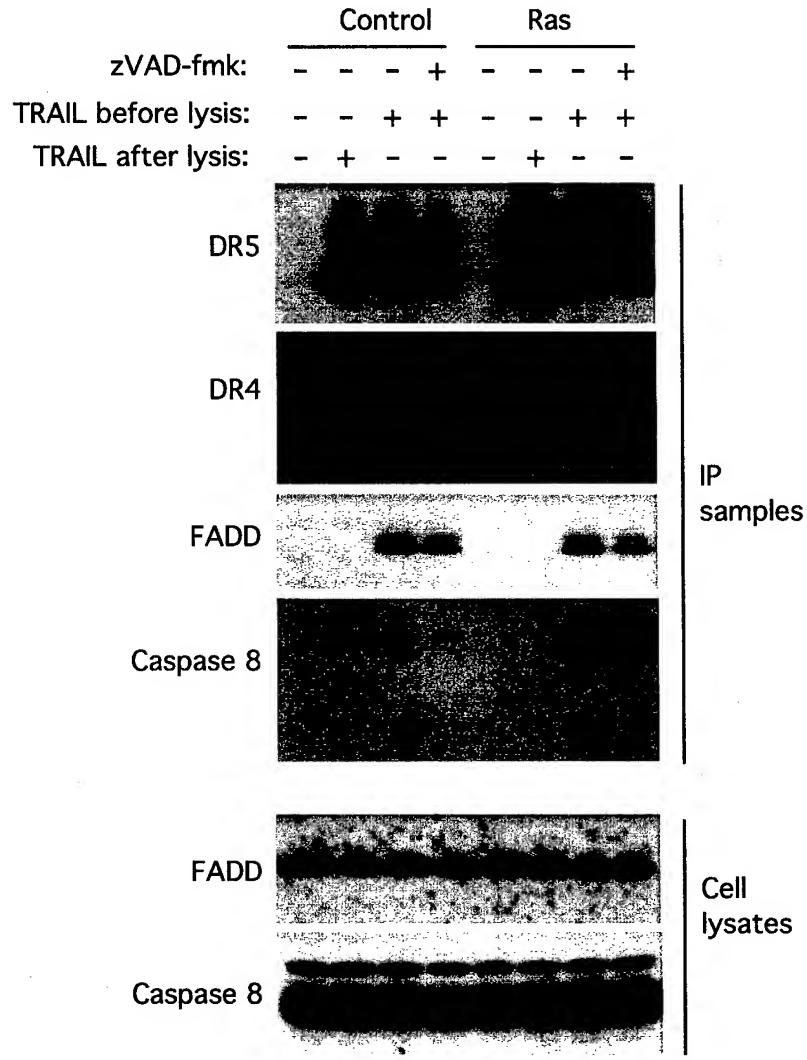

Control
Ras
zVAD-fmk: - - - + - - - +
TRAIL before lysis: - - + + - - + +
TRAIL after lysis: - + - - - + - -
DR5
DR4
FADD
Caspase 8
IP samples
FADD
Caspase 8
Cell lysates

Figure 4

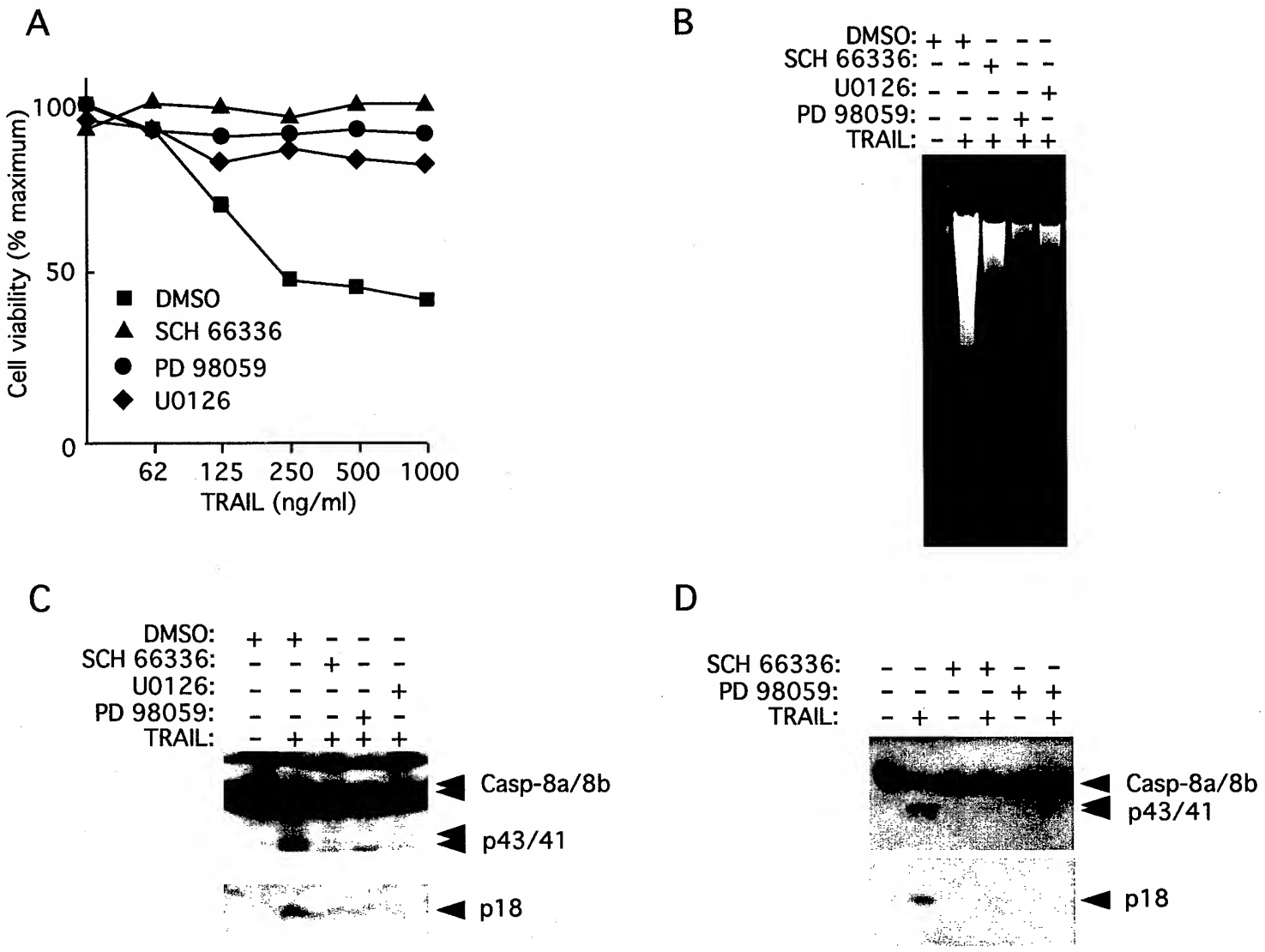
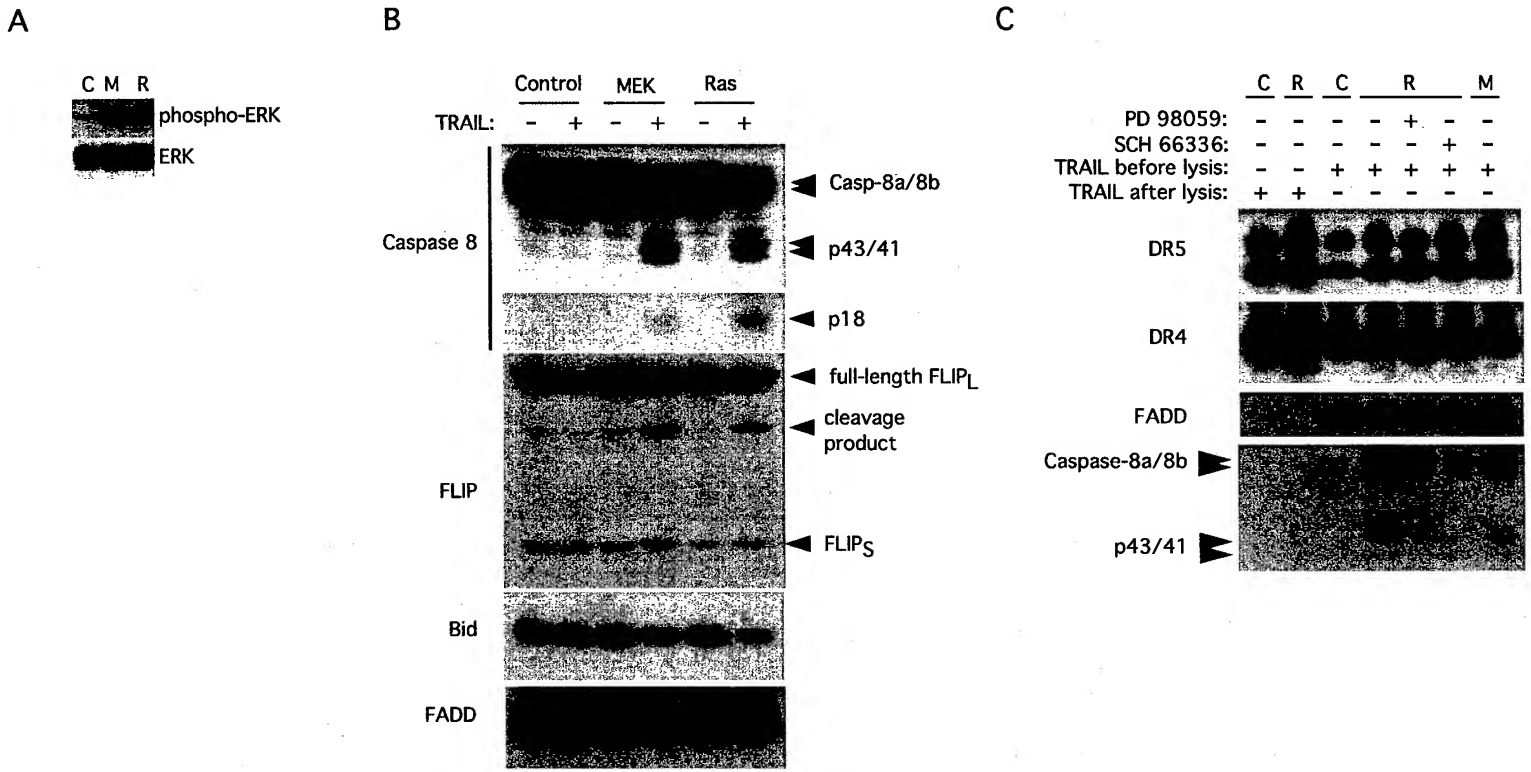

A
100
50
0
Cell viability (% maximum)
DMSO
SCH 66336
PD 98059
U0126
62
125
250
500
1000
TRAIL (ng/ml)
B
DMSO: + + - - -
SCH 66336: - - + - -
U0126: - - - - +
PD 98059: - - - + -
TRAIL: - + + + +
C
DMSO: + + - - -
SCH 66336: - - + - -
U0126: - - - - +
PD 98059: - - - + -
TRAIL: - + + + +
Casp-8a/8b
p43/41
p18
D
SCH 66336: - - + + - -
PD 98059: - - - - + +
TRAIL: - + - + - +
Casp-8a/8b
p43/41
p18

Figure 5


A
C M R
phospho-ERK
ERK
B
Control
MEK
Ras
TRAIL: - + - + - +
Caspase 8
Casp-8a/8b
p43/41
p18
full-length FLIPL
cleavage product
FLIP
FLIPS
Bid
FADD
C
C R C R M
PD 98059: - - - - + - -
SCH 66336: - - - - - + -
TRAIL before lysis: - - + + + + +
TRAIL after lysis: + + - - - - -
DR5
DR4
FADD
Caspase-8a/8b
p43/41